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(54) Title: INHIBITORS OF ROTAMASE ENZYME ACTIVITY

57) Abstract

This invention relates to the method of using neurotrophic pipecolic acid derivative compounds having an affinity for FKBP-type immunophilins as inhibitors of the enzyme activity associated with immunophilin proteins, and particularly inhibitors of peptidyl-prolyl isomerase or rotamase enzyme activity to stimulate or promote neuronal growth or regeneration.

INHIBITORS OF ROTAMASE ENZYME ACTIVITY

Related Application

This application is a continuation-in-part application of U.S. Patent Application Serial No.
5   08/474,072 filed June 7, 1995.

BACKGROUND OF THE INVENTION

1.   Field of the Invention

This invention relates to the method of using neurotrophic pipecolic acid derivative compounds
10   having an affinity for FKBP-type immunophilins as inhibitors of the enzyme activity associated with immunophilin proteins, and particularly inhibitors of peptidyl-prolyl isomerase or rotamase enzyme activity.

15   #### 2.   Description of the Prior Art

The term immunophilin refers to a number of proteins that serve as receptors for the principal immunosuppressant drugs, cyclosporin A (CsA), FK506, and rapamycin. Known classes of immunophilins are
20   cyclophilins, and FK506 binding proteins, such as FKBP. Cyclosporin A binds to cyclophilin while FK506 and rapamycin bind to FKBP. These immunophilin-drug complexes interface with a variety of intracellular signal transduction systems,
25   especially in the immune system and the nervous system.

Immunophilins are known to have peptidyl-prolyl isomerase (PPIase) or rotamase enzyme activity. It has been determined that rotamase activity has a
30   role in the catalyzation of the interconversion of the cis and trans isomer of immunophilin proteins.

Immunophilins were originally discovered and studied in immune tissue. It was initially

postulated by those skilled in the art that
inhibition of the immunophilins rotamase activity
leads to the inhibition of T-cell proliferation,
thereby causing the immunosuppressive action
exhibited by immunosuppressive drugs such as
cyclosporin A, FK506, and rapamycin. Further study
has shown that the inhibition of rotamase activity,
in and of itself, is not sufficient for
immunosuppressant activity. Schreiber et al. *Science*
1990, 250, 556-559. Instead immunosuppression
appears to stem from the formulation of a complex of
immunosuppressant drugs and immunophilins. It has
been shown that the immunophilin-drug complexes
interact with ternary protein targets as their mode
of action. Schreiber et al., *Cell* 1991, 66, 807-815.
In the case of FKBP-FK506 and FKBP-CsA, the drug-
immunophilin complexes bind to the enzyme
calcineurin, inhibiting T-cell receptor signalling
leading to T-cell proliferation. Similarly, the
complex of rapamycin and FKBP interacts with the
RAFT1/FRAP protein and inhibits signalling from the
IL-2 receptor.

Immunophilins have been found to be present at
high concentrations in the central nervous system.
Immunophilins are enriched 10-50 times more in the
central nervous system than in the immune system.
Within neural tissues, immunophilins appear to
influence nitric oxide synthesis, neurotransmitter
release, and neuronal process extension.

Nitric oxide serves several roles in the body.
In the brain, nitric oxide appears to be a
neurotransmitter. It is formed, from arginine, by
nitric oxide synthetase which oxidizes the guanidino
group of arginine forming nitric oxide and
citrulline. Stimulation of the N-methyl-d-aspartate
(NMDA) subtype of glutamate receptors rapidly and
markedly activates nitric oxide synthetase and

stimulates cGMP formation. Inhibition of nitric oxide synthetase with arginine derivatives such as nitroarginine blocks the glutamate induced increase in cGMP levels. Nitric oxide synthetase is a

5 calcium-calmodulin requiring enzyme and N-methyl-d-aspartate receptor activation stimulates nitric oxide synthetase activity because the N-methyl-d-aspartate receptor possesses a calcium channel which is opened by glutamate stimulation, allowing calcium

10 to rush into the cells and activate the nitric oxide synthetase.

Glutamate is a physiologic neurotransmitter. However, when released in excess, glutamate elicits neurotoxicity via N-methyl-d-aspartate receptors.

15 Treatment of cerebral cortical neuronal cultures with glutamate or N-methyl-d-aspartate kills up to 90% of neurons and these effects are blocked by N-methyl-d-aspartate antagonist drugs. This N-methyl-d-aspartate neurotoxicity is thought to be a major

20 contributor to neuronal damage following vascular stroke. Thus, there is a massive release of glutamate following cerebral vascular occlusion and numerous N-methyl-d-aspartate antagonists block stroke damage. Phosphorylation of nitric oxide

25 synthetase inhibits its catalytic activity. By enhancing nitric oxide synthetase phosphorylation, FK506 might functionally inhibit nitric oxide formation and thus block glutamate neurotoxicity. Indeed, low concentrations of FK506 and cyclosporin

30 A both block N-methyl-d-aspartate neurotoxicity in cortical cultures. The mediating role of FKBP is evident, as rapamycin reverses the therapeutic effect of FK506. Presumably FK506, already marketed as an immunosuppressant, could be clinically

35 employed in stroke patients.

FK506 also augments the phosphorylation of growth-associated protein-43 (GAP43). GAP43 is

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involved in neuronal process extension and its
phosphorylation appears to augment this activity.
Accordingly, the effects of FK506 rapamycin and
cyclosporin in neuronal process extension have been
5   examined using PC12 cells. PC12 cells are a
continuous line of neuronal-like cells which extend
neurites when stimulated by nerve growth factor
(NGF).

Surprisingly, it has been found that picomolar
10   concentrations of an immunosuppressant such as FK506
and rapamycin stimulate neurite out growth in PC12
cells and sensory nervous, namely dorsal root
ganglion cells (DRGs). Lyons et al. *proc. Natl.
Acad. Sci. USA*, 1994, 91, 3191-3195. In whole
15   animal experiments, FK506 has been shown to
stimulate nerve regeneration following facial nerve
injury and results in functional recovery in animals
with sciatic nerve lesions.

More particularly, it has been found that drugs
20   with a high affinity for FKBP are potent rotamase
inhibitors and exhibit excellent neurotrophic
effects. Snyder et al., "Immunophilins and the
Nervous System", *Nature Medicine*, Volume 1, No. 1,
January 1995, 32-37. These findings suggest the use
25   of immunosuppressants in treating various peripheral
neuropathies and enhancing neuronal regrowth in the
central nervous system (CNS). Studies have
demonstrated that neurodegenerative disorders such
as Alzheimer's disease, Parkinson's disease, and
30   amyotrophic lateral sclerosis (ALS) may occur due to
the loss, or decreased availability, of a
neurotrophic substance specific for a particular
population of neurons affected in the disorder.

Several neurotrophic factors effecting specific
35   neuronal populations in the central nervous system
have been identified. For example, it has been
hypothesized that Alzheimer's disease results from a

decrease or loss of nerve growth factor (NGF). It
has thus been proposed to treat Alzeheimer's
patients with exogenous nerve growth factor or other
neurotrophic proteins such as brain derived growth
5 ·   factor, glial derived growth factor, ciliary
neurotrophic factor, and neurotropin-3 to increase
the survival of degenerating neuronal populations.
        Clinical application of these proteins in
various neurological disease states is hampered by
10   difficulties in the delivery and bioavailability of
large proteins to nervous system targets. By
contrast, immunosuppressant drugs with neurotrophic
activity are relatively small and display excellent
bioavailability and specificity. However, when
15   administered chronically, immunosuppressants exhibit
a number of potentially serious side effects
including nephrotoxicity, such as impairment of
glomerular filtration and irreversible interstitial
fibrosis (Kopp et al., 1991, *J. Am. Soc. Nephrol.*
20   1:162); neurological deficits, such as involuntary
tremors, or non-specific cerebral angina such as
non-localized headaches (De Groen et al., 1987, *N.
Engl. J. Med.* 317:861); and vascular hypertension
with complications resulting therefrom (Kahan et
25   al., 1989 *N. Engl. J. Med.* 321: 1725).
        The present invention provides non-
immunosuppressive as well as immunosuppressive
pipecolic acid derivative compounds containing small
molecule FKBP rotamase inhibitors which are
30   extremely potent in augmenting neurite outgrowth,
and for promoting neuronal growth, and regeneration
in various neuropathological situations where
neuronal repair can be facilitated including
peripheral nerve damage by physical injury or
35   disease state·such as diabetes, physical damage to
the central nervous system (spinal cord and brain),
brain damage associated with stroke, and for the

treatment of neurological disorders relating to
neurodegeneration, including Parkinson's disease,
Alzheimer's disease, and amyotrophic lateral
sclerosis.

5   SUMMARY OF THE INVENTION
        This invention relates to the method of using
neurotrophic pipecolic acid derivative compounds
having an affinity for FKBP-type immunophilins as
inhibitors of the enzyme activity associated with
10 immunophilin proteins, and particularly inhibitors
of peptidyl-prolyl isomerase or rotamase enzyme
activity.
        A preferred embodiment of this invention is a
method of treating a neurological disorder in an
15 animal, comprising:
            administering to an animal an effective amount
            of a pipecolic acid derivative having an
            affinity for FKBP-type immunophilins to
            stimulate growth of damaged peripheral nerves
20   or to promote neuronal regeneration, wherein
            the FKBP-type immunophilin exhibits rotamase
            activity and the pipecolic acid derivative
            inhibits said rotamase activity of the
            immunophilin.
25   Another preferred embodiment of this invention
is a method of treating a neurological disorder in
an animal, comprising:
            administering to an animal an effective amount
            of a pipecolic acid derivative having an
30   affinity for FKBP-type immunophilins in
            combination with an effective amount of a
            neurotrophic factor selected from the group
            consisting of neurotrophic growth factor, brain
            derived growth factor, glial derived growth
35   factor, cilial neurotrophic factor, and
            neurotropin-3, to stimulate growth of damaged

peripheral nerves or to promote neuronal
regeneration, wherein the FKBP-type
immunophilin exhibits rotamase activity and the
pipecolic acid derivative inhibits said

5   rotamase activity of the immunophilin.
Another preferred embodiment of this invention
is a method of stimulating growth of damaged
peripheral nerves, comprising;
administering to damaged peripheral nerves an

10   effective amount of a pipecolic acid derivative
compound having an affinity for FKBP-type
immunophilins to stimulate or promote growth of
the damaged peripheral nerves, wherein the
FKBP-type immunophilins exhibit rotamase

15   activity and the pipecolic acid derivative
inhibits said rotamase activity of the
immunophilin.
Another preferred embodiment of this invention
is a method of stimulating growth of damaged

20   peripheral nerves, comprising:
administering to damaged peripheral nerves an
effective amount of a pipecolic acid derivative
compound having an affinity for FKBP-type
immunophilins to stimulate growth of damaged

25   peripheral nerves, wherein the FKBP-type
immunophilin exhibit rotamase activity and the
pipecolic acid derivative inhibits said
rotamase activity of the immunophilin.
Another preferred embodiment of this invention

30   is a method for promoting neuronal regeneration and
growth in animals, comprising:
administering to an animal an
effective amount of a pipecolic acid derivative
compound having an affinity for FKBP-type

35   immunophilins to promote neuronal regeneration,
wherein the FKBP-type immunophilins exhibit
rotamase activity and the pipecolic acid

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derivative inhibits said rotamase activity of
the immunophilin.

Yet another preferred embodiment of this
invention is a method for preventing
5   neurodegeneration in an animal, comprising:
administering to an animal an effective amount
of a pipecolic acid derivative having an
affinity for FKBP-type immunophilins to prevent
neurodegeneration, wherein the FKBP-type
10   immunophilin exhibits rotamase activity and the
pipecolic acid derivative inhibits said
rotamase activity of the immunophilin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1

15   FKBP-12 and GAP-43 expression in the facial
nucleus after nerve crush. *In situ* hybridization
comparing the time course of expression of mRNA in
the facial nucleus for FKBP12 (left) and GAP-43
(right). The right facial nucleus is ipsilateral to
20   the crush, and the left side is an unoperated
control (Fig 1B). *In situ* hybridization for FKBP-12
on an untreated control (left) and for calcineurin
Aα,β 7 days following facial nerve crush (right).

Experiments were replicated at least 3 times
25   with similar results.

Figure 2

Localization of FKBP-12 to facial motor neurons
following nerve crush. Bright-field
photomicrographs of *in situ* hybridization for FKBP-
30   12 in motor neurons of the facial nucleus 7 days
after crush (Fig. 2A), and in motor neurons of
control facial nucleus (Fig. 2B).

Figure 3

Upregulation of FKBP-12 mRNA in lumbar spinal
35   cord motor neurons after sciatic nerve crush. *In
situ* hybridization for FKBP-12   7 days after crush

of the right sciatic nerve. Top panel (Fig. 3A)
shows the response of motor neurons in the ventral
horn of lower lumbar spinal cord (indicated by the
arrow). Bright field photomicrographs of
5   corresponding motor neuron pools are shown in the
bottom panels: (Fig. 3B) left side contralateral to
nerve crush, (Fig. 3C) right side ipsilateral to the
nerve crush. This experiment was repeated 3 times
with similar results.

10   Figure 4

Induction of FKBP and FKBP-12 mRNA in the
dorsal root ganglion 1 and 6 weeks after sciatic
nerve crush. Dark-field photomicrographs of
sections through the L4 dorsal root ganglion
15   ipsilateral to sciatic nerve crush processed for
FKBP *in situ* hybridization are shown in the left
panels and for [³H]FK506 autoradiography in the right
panels. These results were replicated 3 times for
each time point.

20   Figure 5

Ricin lesion of the right facial nerve. Nissl
stain (bottom panel, Fig. 5A) reveals extensive
degeneration of motor neurons in the right facial
nucleus with an accompanying glial proliferation 7
25   days following injection of ricin into the facial
nerve. *In situ* hybridization for FKBP mRNA 7 days
after ricin lesion of the facial nerve/nucleus is
shown in the top panel (Fig. 5B). This experiment
was replicated 3 times with similar results.

30   Figure 6

[³H]FK506 binding in segments of sciatic nerve 7
days following crush. The diagram illustrates the 3
mm segments of nerve taken: constrictions indicate
positions of ligatures applied at day 7 for the 6 hr
35   collection time as described in the methods. The
distal ligature site is 10 mm proximal to the
original crush site. Anterograde transport of FKBP

is 124 mm/day.   Data are the means ± S.E.M. (n = 3).

        Figure 7

        Transport of FKBP in the sciatic nerve. Dark-
field photomicrographs of sections through a control
5   (untreated) sciatic nerve and a 7 day sciatic nerve
crush site processed for FKBP-12 *in situ*
hybridization (Fig. 7A, Fig. 7B) and for [^{3}H]FK-506
autoradiography (Fig. 7C, Fig. 7D). Arrows indicate
the sight of the nerve crush. This experiment was
10 repeated 3 times with similar results.

        Figure 8

        Levels of [^{3}H]FK506 binding in PC-12 cells
maintained in the presence or absence of NGF (50
ng/ml).n = 3 for each time point. Bars represent
15 S.E.M.

        Figure 9

        Immunosuppressant mediated enhancement of
neurite outgrowth in PC-12 cells. Hoffman contrast
photomicrographs (64) of cultures grown for 48 hr in
20 the presence of NGF with or without added FK506 or
rapamycin. Fig. 9A: PC-12 cells grown in 1.0 ng/ml
NGF. Fig. 9B: 50 ng/ml NGF. Fig. 9C: 1.0 ng/ml NGF
and 100 nM FK506. Fig. 9D: 1.0 ng/ml NGF and 100 nM
rapamycin. Magnification 200 X.

25   Figure 10

        Effects of FK506 on neurite outgrowth in PC-12
cells. Cultures were treated with varying
concentrations of NGF in the presence or absence or
100 nM FK506, and neurite sprouting was measured at
30 48 hr. Outgrowth was quantitated as described in
Methods by counting cells with neuritic processes
greater than 5 μm. n = 4 separate experiments for
each point and error bars represent SEM.

        Figure 11

35   Concentration-response relationship for FK506
potentiation of neurite outgrowth in PC-12 cells.
Cells were treated for 48 hr with 1 ng/ml NGF and

varying concentrations of FK506. Neurite outgrowth
response was measured as described in Figure 10 and
Methods. n = 4 separate experiments for each data
point *p<.001 Students t test.

5   **Figure 12**

[³H]FK-506 autoradiography on dorsal root
ganglion explant cultures. After 26 days of
cultures with 100 ng/ml NGF the extensive processes
display abundant FKBP associated silver grains.

10   Autoradiographic grains are abolished with 1 μM
unlabeled FK506.

Figure 13

Phase-contrast micrographs of dorsal root
ganglia grown with different substances. Fig. 13A:

15   NGF 100 ng/ml, Fig. 13B: FK506 1 μM, Fig. 13C: FK506
1 μM and anti-NGF antibody, Fig. 13D: No added
growth factor, Fig. 13E: FK506 1pM, Fig. 13F: FK506
1μM. and rapamycin 1 μM. Scale bar is 205 μm. NGF
produces abundant axon outgrowth (Fig. 13A), as does

20   1 μM FK506 (Fig. 13B). The effects of FK506 are
substantially decreased by reducing the
concentration to 1 pM (Fig. 13E). However, neurite
outgrowth with 1 pM FK506 is greater than in its
absence (Fig. 13D). FK506 effects are also

25   diminished by adding anti-NGF antibody to eliminate
the effects of NGF produced by non-neuronal cells in
the cultures. The abundant neurites that occur in
large fascicles in response to NGF (100 ng/ml])
(Fig. 13A) or 1 μM FK506 (Fig. 13B) appear white,

30   while small fascicles or individual neurites appear
black. Non-neuronal cells, Schwann cells and some
fibroblasts, are more evident with 1 pM FK506 (Fig.
13E) or anti-NGF antibody (Fig. 13C) than with 1 μM
FK506 (Fig. 13B). NGF produced by non-neuronal

35   cells in the cultures results in the limited axon
outgrowth seen in cultures with no added growth
factors (Fig. 13D). The large number of refractile

non-neuronal cells, appearing white, tend to
overshadow the few neurites (Fig. 13D). Rapamycin
completely inhibits axon outgrowth in the presence
of FK506 (Fig. 13F). Micrographs are representative
5   of 12-30 ganglia from each experimental condition.
Differences between all experimental groups were
highly reproducible.
Figure 14

Effects of FK506 and rapamycin on NGF-mediated
10   neurite extension in PC12 cells. PC12 cells
(passage 60) were treated with various
concentrations of NGF alone or in the presence of
100 nM FK506, 100 nM rapamycin or 100 nM
WAY-124,466. Neurite outgrowth was measured after
15   96 hours with cells bearing processes longer than
the diameter of the cells scoring positive. n=3
separate experiments for each point and error bars
represent S.E.M.
Figure 15

20   Picomolar concentrations of (A) FK506 and (B)
rapamycin and WAY-124,466 potentiate neurite
extension elicited by NGF (0.5 ng/ml) in PC12 cells.
Low passage PC12 cells were treated for 4 days with
0.5 ng/ml NGF in the presence of various
25   concentrations of FK506 (□), rapamycin ( ) or
WAY-124,466 ( ). Neurite expression was
quantitated as described above in figure 14. The
levels of neurite production in the presence of 0.5
ng/ml NGF (designated L) and 50 ng/ml NGF
30   (designated H) are indicated for comparative
purposes.
Figure 16

Photomicrographs of PC12 cells treated with
immunophilin ligands + 0.5 ng/ml NGF itself or 50
35   ng/ml NGF.
Figure 17

Immunophilin ligands reduce the amount of NGF

required to produce maximal neurite extension in chick sensory ganglia. Whole dorsal root ganglion explants were isolated from day 9-10 chick embryos and cultured in Matrigel-coated 12-well dishes containing L15 medium plus high glucose , with 10% fetal calf serum supplemented with $10\mu M$ Ara C penicillin and streptomycin) at 37°C in a 5% CO_2 environment. Sensory ganglia were treated with 1 ng/ml NGF, 1 ng/ml NGF plus 100 nM FK506 or 100 ng/ml NGF for 48 hr, and neuronal processes were counted and photographed.

Figure 18

FK506, rapamycin, and WAY-124,466 potentiate NGF-dependent neurite production in sensory ganglia. Explants of chick DRG were cultured as described in figure 17 above. FK506, rapam,ycin and WAY-124,466 (100nM each plus or minus 0.1 ng/ml NGF were added to the DRG explant cultures. At 48 hrs., neurite outgrowth was quantitated and the cultures were photographed.

Figure 19

Photomicrograph of Example 111 promoting neurite outgrowth in Chick dorsal root ganglion cultures. The three panels show neurite outgrowth at 1 pM concentration (left panel), 100 pM concentration (center panel), and 100 nM concentration (right panel) of Example 111.

Figure 20

Photomicrograph of Example 17 promoting neurite outgrowth in dorsal root ganglion cultures. The three panels show neurite outgrowth at 1 pM concentration (left panel), 100 pM concentration (center panel), and 100 nM concentration (right panel) of Example 17.

Figure 21

Photomicrograph of Example 102 promoting neurite outgrowth in dorsal root ganglion cultures. The three panels show neurite outgrowth at 1 pM concentration (left panel), 100 pM concentration (center panel), and 100 nM concentration (right panel) of Example 102.

DETAILED DESCRIPTION OF THE INVENTION

The novel neurotrophic pipecolic acid derivative compounds of this invention have an affinity for the FK506 binding proteins such as FKBP-12. When the neurotrophic compounds of the invention are bound to FKBP, they have been found to inhibit the prolyl- peptidyl cis-trans isomerase activity, or rotamase activity of the binding protein and unexpectedly stimulate neurite growth.

The compounds of the present invention can be used in the form of salts derived from inorganic or organic acids and bases. Included among such acid salts are the following: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemissulfate heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalensulfonate, nicotinate, oxalate, pamoate, pectinate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate. Base salts include ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salt with organic bases such as dicyclohexylamine salts, N-

methyl-D-glucamine, and salts with amino acids such
as arginine, lysine, and so forth. Also, the basic
nitrogen-containing groups can be quarternized with
such agents as lower alkyl halides, such as methyl,
5   ethyl, propyl, and butyl chloride, bromides and
iodides; dialkyl sulfates like dimethyl, diethyl,
dibutyl and diamyl sulfates, long chain halides such
as decyl, lauryl, myristyl and stearyl chlorides,
bromides and iodides, aralkyl halides like benzyl
10   and phenethyl bromides and others. Water or oil-
soluble or dispersible products are thereby
obtained.

        The neurotrophic compounds of this invention
can be periodically administered to a patient
15   undergoing treatment for neurological disorders or
for other reasons in which it is desirable to
stimulate neuronal regeneration and growth, such as
in various peripheral neuropathic and neurological
disorders relating to neurodegeneration. The
20   compounds of this invention can also be administered
to mammals other than humans for treatment of
various mammalian neurological disorders.

        The novel compounds of the present invention
are potent inhibitors of rotamase activity and
25   possess an excellent degree of neurotrophic
activity. This activity is useful in the
stimulation of damaged neurons, the promotion of
neuronal regeneration, the prevention of
neurodegeneration, and in the treatment of several
30   neurological disorders known to be associated with
neuronal degeneration and peripheral neuropathies.
The neurological disorders that may be treated
include but are not limited to: trigeminal
neuralgia, glossopharyngeal neuralgia, Bell's Palsy,
35   myasthenia gravis, muscular dystrophy, amyotrophic
lateral sclerosis, progressive muscular atrophy,
progressive bulbar inherited muscular atrophy,

herniated, ruptured or prolapsed invertabrae disk
syndromes, cervical spondylosis, plexus disorders,
thoracic outlet destruction syndromes, peripheral
neuropathic such as those caused by lead, dapsone,
5   ticks, porphyria, or Gullain-Barré syndrome,
Alzheimer's disease, and Parkinson's disease.

For these purposes the compounds of the present
invention may be administered orally, parenterally,
by inhalation spray, topically, rectally, nasally,
10   buccally, vaginally or via an implanted reservoir in
dosage formulations containing conventional non-
toxic pharmaceutically-acceptable carriers,
adjuvants and vehicles. The term parenteral as used
herein includes subcutaneous, intravenous,
15   intramuscular, intraperitoneally, intrathecally,
intraventricularly, intrasternal and intracranial
injection or infusion techniques.

To be effective therapeutically as central
nervous system targets the immunophilin-drug complex
20   should readily penetrate the blood-brain barrier
when peripherally administered. Compounds of this
invention which cannot penetrate the blood-brain
barrier can be effectively administered by an
intraventricular route.

25   The pharmaceutical compositions may be in the
form of a sterile injectable preparation, for
example as a sterile injectable aqueous or
oleaginous suspension. This suspension may be
formulated according to techniques know in the art
30   using suitable dispersing or wetting agents and
suspending agents. The sterile injectable
preparation may also be a sterile injectable
solution or suspension in a non-toxic parenterally-
acceptable diluent or solvent, for example as a
35   solution in 1,3-butanediol. Among the acceptable
vehicles and solvents that may be employed are
water, Ringer's solution and isotonic sodium

chloride solution. In addition, sterile, fixed oils
are conventionally employed as a solvent or
suspending medium. For this purpose any bland fixed
oil may be employed including synthetic mono- or
5   diglycerides. Fatty acids such as oleic acid and
its glyceride derivatives find use in the
preparation of injectables, olive oil or castor
oil, especially in their polyoxyethylated versions.
These oil solutions or suspensions may also contain
10   a long-chain alcohol diluent or dispersant.

The compounds may be administered orally in the
form of capsules or tablets, for example, or as an
aqueous suspension or solution. In the case of
tablets for oral use, carriers which are commonly
15   used include lactose and corn starch. Lubricating
agents, such as magnesium stearate, are also
typically added. For oral administration in a
capsule form, useful diluents include lactose and
dried corn starch. When aqueous suspensions are
20   required for oral use, the active ingredient is
combined with emulsifying and suspending agents. If
desired, certain sweetening and/or flavoring and/or
coloring agents may be added.

The compounds of this invention may also be
25   administered in the form of suppositories for rectal
administration of the drug. These compositions can
be prepared by mixing the drug with a suitable non-
irritating excipient which is solid at room
temperature but liquid at rectal temperature and
30   therefore will melt in the rectum to release the
drug. Such materials include cocoa butter, beeswax
and polyethylene glycols.

The compounds of this invention may also be
administered optically, especially when the
35   conditions addressed for treatment involve areas or
organs readily accessible by topical application,
including neurological disorders of the eye, the

skin, or the lower intestinal tract. Suitable topical formulations are readily prepared for each of these areas.

For ophthalmic use, the compounds can be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, preferably, as solutions is isotonic, pH adjusted sterile saline, either with or without a preservative such as benzylalkonium chloride. Alternatively for the ophthalmic uses the compounds may be formulated in an ointment such as petrolatum.

For application topically to the skin, the compounds can be formulated in a suitable ointment containing the compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, the compounds can be formulated in a suitable lotion or cream containing the active compound suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

Topical application for the lower intestinal tract an be effected in a rectal suppository formulation (see above) or in a suitable enema formulation.

Dosage levels on the order of about 0.1 mg to about 10,000 mg.of the active ingredient compound are useful in the treatment of the above conditions, with preferred levels of about 0.1 mg. to about 1,000 mg. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

It is understood, however, that a specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, and the severity of the particular disease being treated and form of administration.

The compounds can be administered with other neurotrophic agents such as neurotrophic growth factor (NGF), glial derived growth factor, brain derived growth factor, ciliary neurotrophic factor, and neurotropin-3. The dosage level of other neurotrophic drugs will depend upon the factors previously stated and the neurotrophic effectiveness of the drug combination.

Methods and Procedures

Sciatic Nerve Crush

This example demonstrates high levels of FKBP in normal peripheral nerve and that these increase following nerve crush.

If FKBP were physiologically associated with neuronal process extension in the actions of GAP-43, then one might anticipate substantial levels of FKBP in peripheral nerve. Accordingly, we measured [^{3}H]FK-506 binding in rat sciatic nerve, as well as in growth cones isolated from 2-day-old rat pups, and compared values with those of the cerebral cortex and several peripheral tissues.

[^{3}H]FK-506 autoradiography was carried out as described on unfixed sections which were thawed and 'air dried before preincubation for 1 hr in buffer consisting of 50 mM Hepes, 2 mg/ml bovine serum albumin, 5% ethanol, and 0.02% Tween 20 pH 7.4. Sections were then exposed to 1 nM [^{3}H]FK-506 (86.5 Ci/mMol; DuPont-NEN, Boston, MA) for 1 hr at room temperature in preincubation buffer. Non-specific

binding was defined by addition of 1 μM FK-506.
Following incubation, the slides were washed 4 x 5
min in ice cold preincubation buffer and air dried.
The radiolabeled sections were then juxtaposed to
5   tritium-sensitive film or coverslips coated with
Kodak NTB-2 emulsion.

TABLE 1

[³H]FK506 Binding to Sciatic Nerve and Growth
Cones

10   (A) [³H]FK506 Binding in Sciatic Nerve

Tissue	Bmax (pmol/mg protein)
Adult Rat	
Sciatic Nerve	22.1
Cerebral Cortex	38.0
Thymus	9.5
Spleen	8.0
Neonatal Rat	
Forebrain	25.5
Growth Cones	10.2

(B) [³H]FK506 Binding After Sciatic Nerve Crush

protein	Bmax fmol/5mm segment	Bmax pmol/mg
Unoperated	31.8 ± 2.1	21.2 ± 1.4
7-Day Crush	136.5 ± 15.7*	40.1 ± 2.0*

[³H]FK506 binding was assayed as described in
methods. In Table 1A experiments were replicated
three times with less than 10% variation. In Table
1B values are presented as the mean ± S.E.M. (n =
3). *P≤ 0.05 Students' t-test for independent
means.

Of all the tissues examined sciatic nerve

binding levels are the highest, somewhat higher than

those of the cerebral cortex and about 10 x higher

than levels in the thymus and spleen, which contain

FKBP associated with lymphocytes. See Table 1A.

Evidence for a role of FKBP in nerve

regeneration comes from experiments in which we

5   crushed the sciatic nerve of adult rats and 7 days

later measured [^{3}H]FK506 binding in a 5 mm segment

immediately proximal to the nerve crush.

Sprague-Dawley rats (175-200g) were

anesthetized with a mixture of Rompun (12 mk/kg)

10   Ketamine (30 mg/kg). Using aseptic techniques, the

facial nerve was crushed with jewelers forceps 2 x

30 sec 2 mm distal to its exit from the stylomastoid

foramen. Identical procedures were used to crush

the sciatic nerve at the level of the mid-thigh.

15   Total binding in the segment proximal to the

crush was quadrupled compared to control values.

Since total protein is substantially augmented in

the proximal segment, [^{3}H]FK-506 binding per mg

protein is only doubled in the proximal segment.

20 Facial Nerve Crush

This example demonstrates that facial nerve

lesions augment the coincident expression of FKBP

and GAP-43.

Following the crush of the facial nerve, mRNA

25   levels of GAP-43 increase in the facial nerve

nucleus. Utilizing *in situ* hybridization, we

examined mRNA levels of FKBP, GAP-43 and calcineurin

following facial nerve crush.

Rats were perfused transcardially with 150-200

ml ice cold phosphate-buffered saline (PBS) (0.1M,

pH 7.4). Tissues were removed and immediately

frozen in isopentane (-80° C). Cryostat sections 18

5   μm thick) were cut and thaw mounted on gelatin

coated slides.

In situ hybridization was performed as

previously described, using antisense

oligonucleotide probes end labeled with [^{35}S]dATP.

10   For FKBP, three separate oligonucleotides

complementary to the following regions of the cloned

cDNA disclosed by Maki, et al. (1990) Proc. Natl.

Acad.-Sci. USA 87, 5440-5443, and Standaert, R.F.,

et al. (1990) Nature 346, 671-674 and incorporated

15   herein by reference, were used: 70-114, 214-258,

441-485. For GAP-43, three separate antisense

oligonucleotides complementary to nucleotides 961-

1008, 1081-1128, 1201-1248 of the cloned cDNA

disclosed by Rosenthal, A., et al. (187) EMBO J. 6,

20   3641-3646 and incorporated herein by reference were

used. For calcineurin Aα antisense oligonucleotides

complementary to the nucleotides 1363-1410 and 1711-

1758, disclosed by Ito et al. (1989) Biochem.

Biophys. Res. Commun. 163, 1492-1497 and

25   incorporated herein by reference, and for

calcineurin Aβ 1339-1386 and 1569-1616 disclosed by

Kuno, T., et al. (1989) Biochem. Biophys. Res.

Commun. 165, 1352-1358 and incorporated herein by

reference, were used. Sections were thawed and

allowed to dry, then fixed for 5 min in 4% freshly

depolymerized paraformaldehyde in PBS. Following

two rinses in PBS, sections were acetylated with

5   0.25% acetic anhydride in 0.1 M triethanolamine 0.5%

NaCl (pH 8.0), and then dehydrated in graded

alcohols, defatted in chloroform for 5 min,

rehydrated to 95% ethanol and allowed to air dry.

Hybridization was performed overnight at 37° C in

10   buffer containing 50% deionized formamide, 10%

dextran sulfate, 4 x SSC, 1 x Denhardt's solution,

20 mM phosphate buffer, 0.1 mg/ml salmon sperm DNA,

0.1 mg/ml yeast transfer RNA, 10 mM dithiothreitol,

2.0% betamercaptoethanol (BMD), 1.0 mM EDTA and

15   labelled probe (2,000,000 dpm/section). Following

hybridization, sections were rinsed in 1 x SSC, 1.0%

BME for 15 min at room temperature, then twice for

10 min at 55° C air dried and placed on film or

dipped in Kodak NTB-2 emulsion.

20   Striking enhancement of FKBP and GAP-43

expression is observed, while no changes are evident

in calcineurin expression. As early as 24 hr

following facial nerve crush FKBP expression is

increased with peak levels evident a 1-2 weeks,

25   while mRNA concentrations diminish substantially at

3 weeks. Examination under higher magnification

reveals that the increased levels of silver grains

for FKBP mRNA are confined to neuronal cell bodies

24

(Figure 2). Northern blot analysis of the dissected

facial nucleus confirms the increased levels of FKBP

specific mRNA. GAP-43 mRNA levels follow a time

course closely similar to those of FKBP. By

5   contrast, no changes in calcineurin expression are

detected at any of the time points examined.

Total cellular RNA from the dissected facial

nucleus was isolated. Samples of 10 or 20 ug total

RNA were electrophoresed through a 1% agarose, 2.0%

10   formaldehyde gel and transferred to a nylon membrane

in 10 nM NaOH. cDNA probes to FKBP labeled with

[35]dCTP to a specific activity of 1×10^9 cpm/ug by

random priming were hybridized overnight at 42° C in

buffer consisting of 50% formamide, 2 x SSPE, 7%

15   SDS, 0.5% Blotto and 100 ug/ml salmon sperm DNA.

The blots were washed for 20 min at room

temperature, and 2 x 15 min at 65° C in 0.15 x SSC,

0.15% SDS and then exposed to film for 48096 hrs.

On the unlesioned side a modest increase in

20   silver grains compared to control sections are

observed. This is consistent with findings that

contralateral neurons also respond to axotomy.

Following facial nerve crush, rats develop a

facial nerve palsy, which is evident by the lack of

25   whisker movement with functional recovery at 3 weeks

coincident with the completion of nerve

regeneration. In our rats we also observed the loss

of whisker movement following nerve crush with a

return of function at 3 weeks. Thus, the time course of increased expression of GAP-43 and FKBP correlates with the process of nerve regeneration.

Sciatic Nerve Regeneration

5   This example demonstrates alterations in FKBP and GAP-43 associated with sciatic nerve regeneration.

Following sciatic nerve lesions GAP-43 mRNA levels are enhanced in both spinal cord motor

10   neurons and in dorsal root ganglia neuronal cells. In rats subjected to sciatic nerve crush, we observed a striking enhancement in mRNA levels for FKBP in motor neurons at L-4, 5 (Figure 3) and in dorsal root ganglia neuronal cells coincident with

15   the reported enhancement of GAP-43 expression (Figure 4). At high magnification we observed the FKMB mRNS silver grains localized to neuronal cell bodies (Figure 3). We monitored FKBP protein levels by autoradiography of [^{3}H]FK-506 binding under

20   conditions in which it binds selectively to FKBP (Figure 4). Increased FKBP is detected in the primary sensory neurons in the dorsal root ganglia, though no increases are evident in motor neuronal cells following sciatic nerve crush.

25   The association of augmented FKBP expression with regeneration selectively is further supported by experiments with ricin. When injected into peripheral nerves ricin is transported back into the

cell body which is destroyed without associated

nerve regeneration. We injected 0.5 ug rici⬤ R

60, Sigma, St. Louis, MO) into the facial ne

the same site where crushes had been performed ⁡

5   other experiments according to the method of Str

and Kreutzbnerg in 0.5 ul PBS and 0.1% Fast Gree

Streit et al., (1988) *J. Comp. Neurol.* 268, 248-

We conducted *in situ* hybridization localiza

studies for FKBP mRNA at 2, 4 and 7 days followi

10   ricin treatment (Figure 5). No increase in FKBP

mRNA is observed following ricin treatment. Gli

occurs both following ricin treatment and nerve

crush. The failure of FKBP mRNA to increase

following ricin treatment fits with the sele⬤iv

15   neuronal localization of FKBP mRNA in the facial

nucleus.

FKBP Transport in the Sciatic Nerve

This example demonstrates that FKBP is rap⁡

transported in the sciatic nerve.

20   The failure of FKBP protein to increase in

motor neurons following sciatic nerve crush des⁡

the increase in FKBP mRNA suggests that the pro⁡

is rapidly transported out of the cell body int⁡

nerve processes. This fits with out earlier

25   observations that FKBP mRNA is concentrated in

granule cells of the cerebellum which contain 1

levels of FKBP protein, while FKBP protein ⬤e

are highly concentrated in the molecular l⁡ r

the cerebellum associated with the parallel fibers

arising from granule cells. To examine for possible

transport of FKBP, we crushed the sciatic nerve and

7 days later applied ligatures 10 and 20 mm proximal

5   to the crush. Six hr following ligature, we

monitored [³H]FK-506 binding in 3 mm segments

spanning the area of the ligatures. (Figure 6).

For axon transport experiments, classic

ligature techniques were used following the methods

10   of Tetzlaff et al. One week following sciatic nerve

crush two collection ligatures (510 sutures) were

placed on the nerve approximately 10 mm apart with

the distal most ligature positioned 10 mm proximal

to the initial crush site. Six hours later, 5-3 mm

15   segments of the nerve were collected from regions

proximal to, distal to, and between the collection

ligatures as illustrated in Figure 5. The nerve

segments were prepared for [³H]FK-506 binding assays

by homogenizing in 10 volumes of 50 mM Tris-HCl,pH

20   7.4 Homogenates were centrifuged at 15,000 x g for

20 min at 4° C, and supernatants were collected and

assayed for total protein concentration using the

Coomassie blue dye binding assay (Pearce). [³H]FK-

506 binding was carried out as described (4) on

25   aliquots containing 2 ug of total soluble protein in

a final volume of 0.4 ml assay buffer consisting of

50 mM Tris-HCl, pH 7.4, 2 mg/ml bovine serum

albumin, 250 pM [³H]FK-506, and varying

28

concentrations of unlabeled FK-506. Following
incubation at 25° C for 60 min; 0.35 ml was layered
over a 0.8 ml column of LH-20 Sephadex (Pharmacia
LKB) and washed with 0.4 ml of assay buffer. The
5   eluates were collected and counted in a
scintillation counter.

Results are shown in Figure 5. [^{3}H]FK-506
binding levels are highest in the segment just
proximal to the ligature 20 cm from the crush, being
10   almost quadruple levels in the other segments.
Based on the levels of [^{3}H]FK-506 binding in segments
A-D, we calculated the rate of anterograde transport
for FKBP. This rate of 240 mm per day is
essentially the same as transport rates for GAP-43
15   representing the most rapid transport rates for
neuronal proteins.

To visualize the accumulation of FKBP following
nerve crush, we applied a loose ligature to mark the
site of crush of the sciatic nerve and conducted *in*
20   *situ* hybridization for FKBP mRNA as well
autoradiography for [^{3}H]FK-506 binding (Figure 7).
Most FKBP mRNA and [^{3}H]FK-506 binding accumulate
immediately proximal to the crush. These levels are
considerably higher than in control uncrushed
25   sciatic nerve. Examination of the *in situ*
hybridization an autoradiography preparations at
high magnification reveals silver grains associated
with neuronal fibers. There are also silver grains

localized to cells whose identity we could not

determine definitively, so that they may be Schwann

cells, macrophages or fibroblasts.

FKBP in PC12 Cells

5   This example demonstrates that PC-12 cells

contain FKBP and that FKBP levels are enhanced by

nerve growth factor. We examined PC-12 cells for

the presence of FKBP by monitoring the binding of

[^{3}H]FK-506 to cells under basal conditions and

10   following treatment with nerve growth factor (NGF).

Levels of FKBP in PC-12 cells were obtained

from Scatchard analysis of [^{3}H]FK-506 binding curves.

Cultures were scraped from the culture wells and

homogenized in 10 volumes of 50 mM Tris-HCl, pH 7.4,

15   1 mM EDTA, 100 μg/ml phenylmethylsulfonylfluoride

and centrifuged at 40,000 x g for 20 min at 4° C.

Protein was determined by the Coomassie blue dye

binding assay using bovine serum albumin as a

standard. Binding of 250 pM [^{3}H]dihydro FK506 (86.5

20   Ci/mmol, DuPont/NEN) was assessed for samples

containing 5 μg soluble protein in a final volume of

0.4 ml assay buffer containing 50 mM Tris-HCl, pH

7.4, 2 mg/ml BSA and varying concentrations of

unlabeled FK506. After 60 min incubation at 25° C,

25   0.35 ml was layered over a 0.8 ml column of LH-20

Sephadex (Pharmacia LKB), pre-equilibrated with

assay buffer. The column was further washed with

0.4 ml of assay buffer, the eluates collected, mixed

with Formula 963 (DuPont/NEN) and counted in a
Beckman scintillation counter. Specific binding was
determined by subtracting binding obtained in the
presence of 1 μM unlabeled FK506 from total [³H]FK506
5   bound.

Results are shown in Figure 8. [³H]FK506 binds
saturably to untreated PC-12 cell homogenates. In
typical experiments about 1,000 cpm are bound while
nonspecific binding in the presence of 1 μM FK506 is
10 about 150 cpm. Fifty percent inhibition of [³H]FK506
binding occurs with 1-2 nM FK506 indicating that the
binding sites correspond to authentic FKBP.
[³H]FK506 binding increases markedly following NFG
treatment. Significant increases are evident by 10-
15 15 hr. Binding triples by 20 hr and a modest
further increase is evident at 100 hr.
Increased Neurite Extension in PC12 Cells

This example demonstrates that FK506 and
rapamycin increase neurite extension in PC-12 cells.
20   PC-12 cells were maintained at 37° C, 5% CO_2, in
Dulbecco's modified Eagle medium (DMEM) supplemented
with 10% heat-inactivated horse serum and 5% heat-
inactivated fetal bovine serum. For differentiation
in NGF, cells were plated at 1 x 10⁵ in 35 mm culture
25 wells coated with rat tail collagen at 5 μg/cm², and
allowed to attach before replacing the media with
DMEM supplemented with 2% fetal horse serum, NGF
and/or FK506 of rapamycin. For quantitation of

neurite outgrowth, random photographs were made (3-4

per well), and process bearing neurons were counted

with processes being greater than 5 μm.

Experimental conditions were unknown by the

5   photographer and cell counter. Four separate

experiments were performed in duplicate for each

data point presented. Neurites were identified and

counted from approximately 100 cells per photograph.

Thus, neurites from 1200 - 1600 cells were counted

10   per data point.

As observed, NGF potently stimulates neurite

outgrowth with half-maximal stimulation at 1 ng/ml

and maximal augmentation at about 50-100 ng/ml

(Figs. 9, 10). FK506 (100 nM) markedly augments the

15   effect of NGF by increasing sensitivity to NGF.

Thus, FK506 reduces by 20-50 fold the NGF

concentration needed to elicit maximal outgrowth.

Half maximal outgrowth in the absence of FK506

occurs at 5 ng/ml NGF and in the presence of FK506

20   at 0.1 ng/ml NGF. At maximal concentrations of NGF

(10-100 ng/ml), FK506 fails to produce additional

neurite outgrowth.

FK506 is extremely potent in its neurotrophic

effects. In the presence of a submaximal

25   concentration of NGF (1 ng/ml) FK506 at 1 nM elicits

the same maximal outgrowth observed with 50 ng/ml

NGF (Fig. 11). Half maximal effects of FK506 occur

at approximately 100 pM. In the absence of NGF,

FK506 fails to elicit neurite outgrowth (Fig. 10).

Rapamycin is a potent immunosuppressant which is not thought to act through calcineurin but which may influence other phosphorylation cascades.

5   Rapamycin potently blocks actions of FK506 that occur through FKBP and calcineurin presumably by acting as an FK506 antagonist at FKBP. Rapamycin (1 μM) fails to block the neurotrophic actions of FK506. Instead, rapamycin is itself neurotrophic

10   providing major neurite outgrowth at 1 nM. Rapamycin and FK506 seem to be acting via different mechanisms. Thus, rapamycin augments the number of processes as well as their length, while FK506 primarily increases neurite length. Moreover,

15   effects of FK506 and rapamycin appear to be additive.

Dorsal Root Ganglia

This example demonstrates that FK506 is neurotrophic for sensory ganglia. We examined the

20   action of FK506 on primary cultures of dorsal root ganglia from rats at embryonic day 16.

Stage E16 embryos were removed from pregnant Sprague-Dawley rats and the dorsal root ganglia dissected. Whole ganglia explants were cultured in

25   collagen-coated 35 mm dishes (Falcon) using N2 medium (Dulbecco's Modified Eagle medium and Ham's F12 medium mixed 1:1 and supplemented with progesterone, selenium, insulin, putrescine,

glucose, and penicillin-streptomycin) at 37° C in a
15% CO_2 environment. Sensory ganglia were treated
with various concentrations of NGF and/or FK506 or
rapamycin or anti-NGF antibody. Ganglia were

5   observed every 2-3 days under phase-contrasting
using an Olympus IMT-2 inverted microscope, and
measurements of axon length were made. The axonal
field of each ganglion was divided into four
quadrants, and the length of the longest axons in

10   each quadrant was measured in microns using an eye-
piece micrometer. The average of these measurements
was taken as the axon length for the ganglion.

For [³H]FK506 autoradiography, dorsal root
ganglia cultures were grown on chamber slides coated

15   with collagen, 5 μg/cm². Cultures were fixed on the
slide with ice cold 4.0% freshly depolymerized
paraformaldehyde in 0.1 M sodium phosphate buffer,
pH 7.4, for 1 hr, then washed two times with
phosphate buffered saline. Fixed cultures were

20   labeled with [³H]FK506 by pre-incubating the slides
in a buffer consisting of 50 mM Hepes, 2 mg/ml
bovine serum albumin, 0.02% Tween-20 pH 7.4. This
was followed by incubation in the same assay buffer
containing 1 nM [³H]FK506. Non-specific binding was

25   determined by adding 1 μM unlabeled FK506. The
slides were then rinsed 4 x 5 min prior to drying,
and juxtaposed to tritium-sensitive film for 10
days.

Autoradiography of [^{3}H] FK506 binding sites reveals substantial levels of FKBP associated silver grains in these ganglia (Fig. 12). At 1 μM unlabeled FK506, autoradiographic grains are

5   abolished indicating the specificity of binding. As reported previously, NGF (100 ng/ml) markedly increases the number and length of ganglia processes (Fig. 13). FK506 (1 μM) alone produces a similar neurotrophic effect, while as little as 1 nM FK506

10   produces a noticeable increase in growth. Rapamycin (1 μM) which acts as an FK506 antagonist, completely blocks the effects of FK506 (1 μM), thus the action of FK506 displays a drug specificity characteristic of FKBP.

15   Whereas FK506 fails to stimulate neurite outgrowth in PC-12 cells in the absence of added NGF, in sensory ganglia FK506 alone is neurotrophic. Schwann cells in the ganglia can fabricate NGF, and the production of NGF by Schwann cells is regulated

20   by a protein phosphorylation event. To ascertain whether the actions of FK506 alone involve potentiation of endogenous NGF, we examined the influence of antibodies to NGF (Fig. 13). Anti-NGF markedly reduces the neurotrophic effects of FK506

25   (1 μM). The anti-NGF is not acting in a toxic fashion as we observe no morphologic evidence of toxicity in the cells exposed to anti-NGF in the presence or absence of added NGF.

FK506 is extremely potent in stimulating neurite outgrowth. As little as 1 pM FK506 produces detectable augmentation. Progressively greater outgrowth occurs at 0.1 and 10 nM FK506 (data not shown), while maximal outgrowth requires 1 μM FK506.

The time course of neurite outgrowth is similar at all concentrations of NGF and FK506. Some outgrowth is evidence by 1 day, while growth begins to plateau at about 5-6 days.

FK506 neurotrophic effects involve FKBP (FK506 binding protein) in sensory ganglia since the effects of FK506 are reversed by low concentrations of rapamycin, a known antagonist of FK506 at FKBP. The failure of rapamycin to block FK506 effects in PC-12 cells probably reflects the separate stimulatory effects of rapamycin. Mechanisms for rapamycin stimulation of neurite outgrowth in PC-12 cells are not immediately evident. Its immunosuppressant actions are thought to involve different mechanisms than those of FK506. Rapamycin can inhibit S6 kinase which phosphorylates the S6 ribosomal subunit. Rapamycin also inhibits phosphatidylinositol-3-kinase.

Protein kinase C (PKC) -mediated phosphorylation has been implicated in process outgrowth during neuronal regeneration. Other evidence suggests inhibitory effects of PKC in neuronal process extension.

GAP43 is a prominent calcineurin substrate highly concentrated in neurites and its phosphorylation is regulated by FKBP. GAP43 may not be directly involved in neurite extension, as PC-12

5   cell lines with low levels of GAP43 display normal neurite outgrowth. However, GAP43 and its phosphorylation may be involved in targeting neurites, as levels of phosphorylated GAP43 are increased when neurites approach their targets.

10   Phosphorylation of GAP-43 may also influence mobilization of Ca^2 that regulates neurite extension. Phosphorylated GAP-43 inhibits phosphatidyl inositol bis-phosphate formation, which should diminish level of inositol 1,4,5-triphosphate and associated Ca^{2+}

15   release. In addition, phosphorylation of GAP-43 decreases its affinity for calmodulin with the resultant free calmodulin available to bind Ca^{2+}.

Immunophilins may act at sites besides calcineurin which affect Ca^{2+} that regulates neurite

20   outgrowth. FKBP binds to the ryanodine receptor, which is a CA^{2+} release channel. In skeletal muscle sarcoplasmic reticulum FK506 dissociates FKBP from the ryanodine receptor to facilitate the Ca^{2+} induced Ca^{2+} release mechanism. In addition, FK506 acts at

25   other sites including FKBP25 steroid receptors and other unidentified targets such as those related to FKBP13. Thus other potential mechanisms may play some role in neurite extension.

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Non-Immunosuppressive and Immunosuppressive Ligands

of Immunophilins Stimulate Neurite Outgrowth in PC-

12 Cells

In the present study we have examined in detail

5   influences of a number of ligands of the

immunophilins upon neurite extension in PC-12 cells

and in intact chick sensory ganglia. We report that

non-immunosuppressive as well as immunosuppressive

ligands are extremely potent in augmenting neurite

10   outgrowth in both PC-12 cells and sensory ganglia.

In our earlier study we found that

immunosuppressants stimulate neurite outgrowth in

PC-12 cells by increasing the potency of nerve

growth factor (NGF) about 10-fold (Lyons et. al.,

15   1994). In the absence of added NGF no neurotrophic

effects are observed. In the present study we

evaluated effects of the immunosuppressant drugs

FK506 and rapamycin on PC-12 neurite outgrowth in

the presence of 0.1 - 100 ng/ml of NGF.

20   In the absence of added NGF, none of the drugs

stimulate neurite outgrowth. At 0.1 ng/ml NGF alone

produces a small increase in neurite extension only

about 15% of maximal effects that are evident at

50 ng/ml (Figure 14). Rapamycin stimulates neurite

25   outgrowth to a greater extent than the other drugs,

with a 3-4 fold stimulation at 0.1 - 0.5 ng/ml NGF.

The extent of augmentation elicited by rapamycin

decreases with higher concentration of NGF and is

not statistically significant at 5-50 ng/ml NGF.
FK506 also is neurotrophic with effects most
apparent at lower NGF concentrations and a maximal
2.5-fold enhancement of neurite outgrowth evident at

5   0.5 ng/ml NGF.

There are three principal structural classes of
immunosuppressant drugs related in structure to
cyclosporin A, FK506, and rapamycin. Though FK506
and cyclosporin A bind to distinct immunophilin

10   proteins, they both act as immunosuppressants by
inhibiting calcineurin. Rapamycin bind with very
high affinity to FKBP-12, but the drug-immunophilin
complex does not in turn bind to calcineurin.
Instead, immunosuppressant actions result from the

15   rapamycin-FKBP-12 complex binding to a recently
identified and cloned protein designated RAFT-1
(rapamycin and FK506 target) and also designated
FRAP (Sabatini and Snyder, 1994; Brown et. al.,
1994; Chen et. al., 1994). Because rapamycin binds

20   potently to FKBP-12 but does not inhibit
calcineurin, it can serve as an antagonist to FK506.
There exist non-immunosuppressive derivatives of
rapamycin. One of these, WAY-124,466, a triene
derivative of rapamycin, binds with high affinity to

25   FKBP-12 and inhibits rotamase activity, but is
devoid of immunosuppressant actions. Cyclosporin A
is a large cyclic undecapeptide. The mere addition
of a methyl group to an alanine at the 6 position

results in an agent that does not inhibit

calcineurin and lacks immunosuppressive effects,

though it inhibits the rotamase activity of

cyclophilin to a similar extent as cyclosporin A (Me

5   CsA ref).

        To ascertain whether immunosuppressant activity

is required for neurotrophic actions, we compared

the neurotrophic effects of FK506, rapamycin and

cyclosporin A with non-immunosuppressant

10   WAY-124,466, evaluating a wide range of

concentrations on PC-12 cells (Figs. 15, 16). All

studies were done in the presence of 0.5 ng/ml NGF.

As observed previously, FK506 very potently

stimulates neurite extension with half-maximal

15   stimulation at 0.5 nM and maximal effects at 5-100

nM.

        Rapamycin is the most potent agent examined and

produces the greatest maximal level of neurite

extension. In repeated experiments 50% of maximal

20   extension is evident at about 0.2-0.4 nM while

maximal effects are evident at about 10-100 nM.

Maximal neurite extension with rapamycin is

comparable to maximal effects of 50 ng/ml NGF. WAY-

124466 also is neurotrophic, but is less potent and

25   produces a lesser maximal effect than rapamycin.

Half-maximal stimulation with WAY-124,466 occurs at

about 10 nM and maximal effects occur at 100-1,000

nM. Thus, rapamycin is about 100-fold more potent

40

than WAY-124,466, resembling its 40-fold greater

potency in binding to FKBP-12 (Table II).

Cyclosporin A is substantially less poten' ha

FK506 or rapamycin in stimulating neurite outgrowt:

5   corresponding to its substantially lesser potency

inhibiting rotamase activity. Fifty percent maxim

stimulation of neurite outgrowth with cyclosporin

is evident at 50 nM with maximal effects at 100 nM

and a decrease in neurite outgrowth at higher

10   concentrations of cyclosporin A. Maximal

stimulation with cyclosporin A is about 60% of

effects of 50 ng/ml NGF.

The general pattern of process extension is

similar with the various immunophilin ligands and

15   with NGF. At concentrations that elicit 50% of

maximal effects, NGF (1-5 ng/ml) 40-50% of ce__s

extend processes at least as long as the cell bod:

while 15% extent longer processes, up to 3-5 time:

the length of the cell body. The pattern is fair:

20   similar with the various drugs examined. Rapamyc

and WAY-124,466 tend to result in a greater numbe

of processes per cell than FK506. Cyclosporin A

tends to be intermediate in terms of numbers or

processes.

25   Nerve Extension Elicited in Chick Dorsal Ro

Ganglia by Non-Immunosuppressive and

Immunosuppressive Ligands of Immunophilins

In our previous study, we observed neur ro:

effects of immunosuppressant drugs in explants of

rat dorsal root ganglia with significant

augmentation in nerve outgrowth observed with FK506

concentrations as low as 1 picomolar (Lyons et. al.,

5   1994). In the rat ganglia neurotrophic effects were

observed with FK506 even in the absence of NGF. In

the present study we have employed explants of chick

dorsal root ganglia, which are easier to employ in

in studies of nerve outgrowth. In the absence of

10   added NGF, we observe minimal effects of

immunophilin ligand drugs. The chick cells are more

sensitive to NGF than PC-12 cells so that we employ

0.1 ng/ml NGF to produce minimal neurite outgrowth

and to demonstrate neurotrophic actions of

15   immunophilin ligands (Fig. 17,18).

Dorsal root ganglion were dissected from chick

embryos of ten day gestation. Whole ganaglion

explants were cultured on thin layer Matrigel-coated

12 well plates with Liebovitz L15 plus high glucose

20   media supplemented with 2mM glutamine and 10% fetal

calf serum, and also containing 10 μM cytosine β-D

arabinofuranoside (Ara C) at 37°C in an environment

containing 5% CO_2. Twenty-four hours later, the

DRG's were treated with various conentrations of

25   nerve growth factor, immunophilin ligands or

combinations of NGF plus drugs. Forty-eight hours

after drug treatment, the ganglia were visualized

under phase contrast or Hoffman Modulation contrast

with a Zeiss Axiovert inverted miocroscope.
Photomicrographs of the explants were made, and
neurite outgrowth was quantititated. Neurites
longer than the DRG diameter were counted as

5   positive, with total number of neurites quantitated
per each experimentla condition. Three to four DRGs
are cultured per well, and each treatment was
performed in duplicate.

The relative potencies of the various

10   immunophilin ligands in stimulating nerve outgrowth
in the ganglia are similar to their relative
potencies in PC-12 cells. Thus, rapamycin is the
most potent agent with an EC_{50} of __1nM__, __10__-fold
more potent than WAY-124,466, while FK506 displays

15   an EC_{50} of __1-2nM__.

The maximal increase in the number of
processes, their length and branching is quite
similar at maximally effective contractions of the
immunophilin ligands and of NGF (100 ng/ml). With

20   progressively increasing concentrations of the
various drugs, one observes a larger number of
processes, more extensive branching and a greater
length of individual processes.

We evaluated the potencies of drugs in binding

25   to FKBP-12 by examining inhibition of ^{3}H-FK506
binding to recombinant FKBP-12. There is a striking
parallel between affinities of drugs for FKBP-12 and
their potencies in stimulating neurite outgrowth and

inhibiting rotamase activity. Clearly, stimulation of nerve outgrowth is unrelated to calcineurin inhibition. Calcineurin inhibition fits well with immunosuppressant actions, WAY-124,466 is not
5 immunosuppressive and fails to inhibit calcineurin. Rapamycin is a potent immunosuppressant, but the rapamycin-FKBP-12 complex binds to RAFT-1 to initiate immunosuppressive processes (Sabatini and Snyder, 1994; Snyder and Sabatini, 1995). The
10 results are set forth in Table 2.

Table 2   IMMUNOPHILIN LIGAND NEUROTROPHISM PARALLELS INHIBITION
                         OF ROTAMASE, NOT CALCINEURIN

Drug	$[^3H]$-FK506-FKBP12 (IC_{50})	Calcineurin Inhibition	Rotamase	Neurite (K_I)	Outgrowth (ED_{50})
15	FK506	0.6 nM	YES 0.4 nM		0.5 nM
	Rapamycin	0.5 nM	NO	0.2 nM	0.5 nM
	WAY-124466	10.0 nM	NO	12.0 nM	10 nM
	Cyclosporin A (CsA)	None	YES 20 nM		50 nM

20   We compared the ability of non-immunosuppressive immunophilin ligands to promote neurite outgrowth in chick dorsal root ganglion explant cultures (Table 3). Each of these compounds is incapable of inhibiting calcineurin, but they interat with the immunophilin FKBP-
25   12 to inhibt it's rotamase activity with the various inhibitory constants listed in Table 3. The ability of these compounds to promote neurite outgrowth in the DRG's correlates well with their ability to inhibit the rotamase activity of FKBP-12.

44

Table 3 IMMUNOPHILIN LIGAND NEUROTROPHISM PARALLELS INHIBITION
OF ROTAMASE, NOT CALCINEURIN

Drug	$[^3H]$-FK506-FKBP12 (IC_{50})	Calcineurin Inhibition	Rotamase (K_i)	Neurite Outgrowth (ED_{50})
Example 12	8 μM	NO	250 nM	300 nM
Example 13	4 μM	NO	25 nM	80 nM

The very close correlation between the potencies of

drugs in binding to immunophilins, inhibiting their

rotamase activity and stimulating neurite outgrowth

implies that inhibition of rotamase activity is

responsible for neurotrophic effects of the drugs. The

extraordinarily high potency of the drugs in stimulating

neurite outgrowth and in binding to immunophilins makes

it most unlikely that any other target could account for

the neurotrophic effects. It is conceivable that a

biological activity of immunophilins other than rotamase

could be influenced by the drugs to mediate neurotrophic

actions. However, no such activity has yet been

reported.

Because of the extraordinary potency of the drugs

and the close correlation between rotamase inhibition and

neurotrophic actions, we conclude that rotamase

inhibition is likely involved in neurotrophic effects. A

number of proteins have been reported as substrates for

the rotamase activity of immunophilins including collagen

(Steinmann et. al., 1991) and transferrin (Lodish and

King, 1991). Recently highly purified preparations of

ryanodine receptor and the IP-3 receptor, prominent

intracellular calcium channels have been reported to

exist in a complex with FKBP-12. Dissociation of FKBP-12 from these complexes causes the calcium channel to become "leaky" (Cameron et. al., 1995) Calcium fluxes are involved in neurite extension so that the IP-3 receptor and the

5 ryanodine receptor might be involved in the neurotrophic effects of drugs. Since the drugs bind to the same site on FKBP-12 as the IP-3 receptor or the ryanodine receptor, one would have to postulate that the drugs displace the channels from FKBP-12. No interaction between these calcium channels

10 in cyclophilin has been reported so that this model would not explain the neurotrophic actions of cyclosporin A.

The neurotrophic actions of the drugs studied here are exerted at extremely low concentrations indicating potencies comparable to those of neurotrophic proteins such as brain

15 derived growth factor, neurotropin-3 and neurotrophic growth factor.

The following examples are illustrative of preferred embodiments of the invention and are not to be construed as limiting the invention thereto. All polymer molecular

20 weights are mean average molecular weights. All percentages are based on the percent by weight of the final delivery system or formulation prepared unless otherwise indicated and all totals equal 100% by weight.

Illustrative pipecolic acid derivative compounds which

25 can be used for the purposes of this invention include:

46

EXAMPLE 1
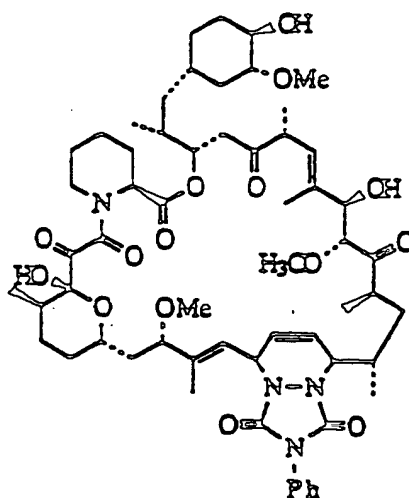
Way-124,466

This exemplary pipecolic acid derivative compound is disclosed by Ocain et al., *Biochemical and Biophysical Research Communications*, Vol. 192, No. 3, 1993. The compound was synthesized at Wyeth-Ayerst by Dr. Phil Hughes by reaction of 4-phenyl-1,2,4-triazoline-3,5-dione with rapamycin.

EXAMPLE 2

47
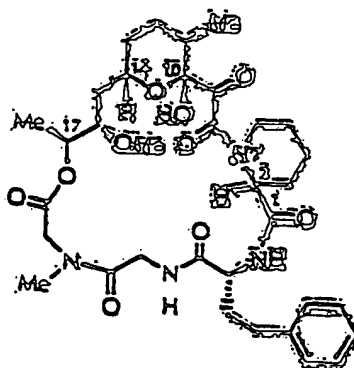
This pipecolic acid derivative compound is disclosed by Chakraborty et al., *Chemistry and Biology*, March 1995, 2:157-161.

EXAMPLES 3-5

n
1
2
3
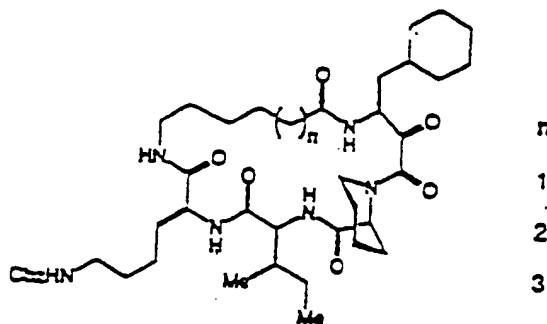
Exemplary pipecolic acid derivative compounds are disclosed by Ikeda et al., *J. Am. Chem. Soc.* 1994, 116, 4143-4144, and are incorporated herein by reference.

EXAMPLES 6-9

Exemplary pipecolic acid derivative compounds are disclosed by Wang et al., *Bioorganic and Medicinal Chemistry Letters*, Vol. 4, No. 9, pp. 1161-1166, 1994, particularly compounds 2a-2d and are incorporated herein by reference.

EXAMPLE 10
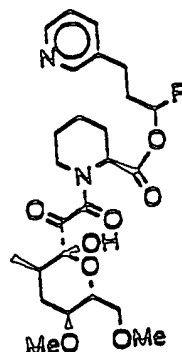
        This exemplary pipecolic acid derivative, compound

10, is disclosed by Birkenshaw et al., *Bioorganic &

Medicinal Chemistry Letters*, Vol. 4, No. 21, pp. 2501-

2506, 1994, and is incorporated herein by reference.

EXAMPLES 11-21

        Exemplary pipecolic acid derivative compounds are

disclosed by Holt et al., *J. Am. Chem. Soc.*, 1993, 1 ,

9925-9938, particularly compounds 4-14, and are

incorporated herein by reference.

EXAMPLES 22-30

        Exemplary pipecolic acid derivative compounds

are disclosed by Caffery et al., *Bioorganic & Medicinal*

Chemistry Letters, Vol. 4, No. 21, pp. 2507-2510, 1994,

and are incorporated herein by reference.

EXAMPLE 31
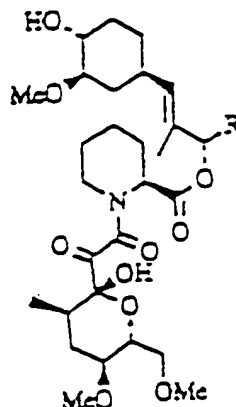
This exemplary pipecolic acid derivative, compound 31, is disclosed by Teague et al., *Bioorganic & Medicinal Chemistry Letters*, Vol. 3, No. 10, pp. 1947-1950, 1993 and is incorporated herein by reference.

EXAMPLES 32-34

Exemplary pipecolic acid derivative compounds are disclosed by Yamashita et al., *Bioorganic & Medicinal Chemistry Letters*, Vol. 4., No. 2, pp. 325-328, 1994, particularly, compounds 11, 12, and 19, and are incorporated herein by reference.

EXAMPLE 35-55

Exemplary pipecolic acid derivatives are disclosed by Holt et al., *Bioorganic & Medicinal Chemistry Letters*, Vol. 4, No. 2, pp. 315-320, 1994, particularly, compounds 3-21, and 23-24, and are incorporated herein by reference.

50

EXAMPLES 56-68

Exemplary pipecolic acid derivative compounds are disclosed by Holt et al., *Bioorganic & Medicinal Chemistry Letters*, Vol. 3, No. 10, pp. 1977-1980, 1993,

5   particularly compounds 3-15 and are incorporated by reference herein.

EXAMPLES 69-83

Exemplary compounds of the present invention are disclosed by Hauske et al., *J. Med. Chem.* 1992, 35, 4284-

10   4296, particularly compounds 6, 9-10, 21-24, 26, 28, 31-32, and 52-55, and are incorporated herein by reference.

EXAMPLE 84
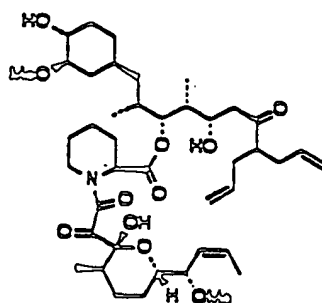
SLB506

This exemplary pipecolic acid derivative is

15   disclosed by Teague et al., *Bioorganic & Med. Chem. Letters*, Vol. 4, No. 13, pp. 1581-1584, 1994, and is incorporated herein by reference.

51

EXAMPLES 85-89

Exemplary pipecolic acid derivative compounds are
disclosed by Stocks et al., *Bioorganic & Med. Chem.
Letters*, Vol. 4, No. 12, pp. 1457-1460, 1994,
5   particularly compounds 2, 15-17 and are incorporated
herein by reference.

EXAMPLES 90-111

Additional exemplary pipecolic acid derivatives are
described in Scheme 10, Tables 1-5.

10   SCHEME 01
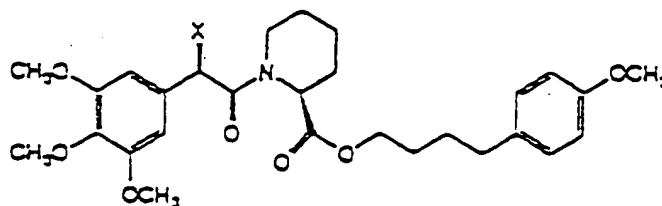
EXAMPLE/COMPOUND	STRUCTURE
6	$X = H_2$
7	$X = CH_2$
8	$X = H, CH_3$
9	$X = O$

15

SUBSTITUTE SHEET (RULE 26)
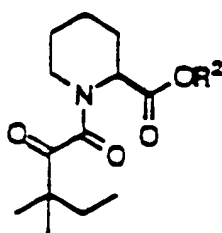
SCHEME 2

EXAMPLE/COMPOUNDS No.		R₂
	11	
	12	
5	13	
	14	
	15	
	16	
	17	
10	18	

SCHEME 3

EXAMPLE/COMPOUND NO.	STRUCTURE
19	
20	
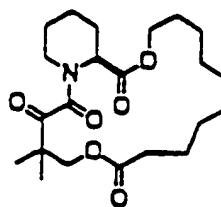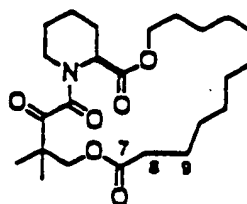
5

21
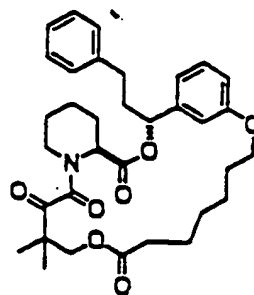
Scheme 4

Table 1
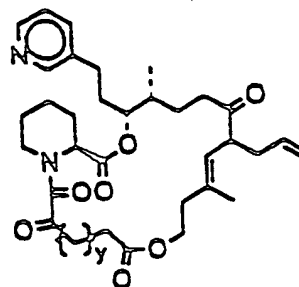
Example/Compound No.	Structure
24	y=1
23	y=2
24	y=3

Table 2
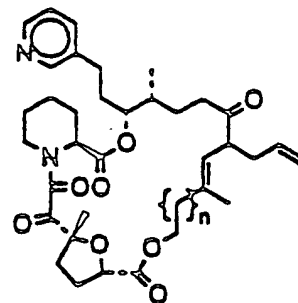
Example/Compound No.	Structure
25	n=1
26	n=2
27	n=3

Table 3.
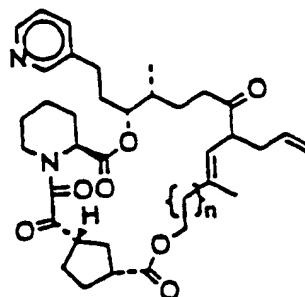
Example/Compound No.	Structure
28	n=1
29	n=2
30	n=3
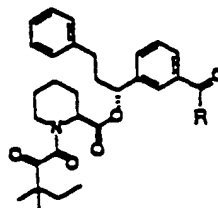
SCHEME 5

EXAMPLE/COMPOUND No.	STRUCTURE
32	R=phenyl
33	R=N(allyl)$_2$
34	

56
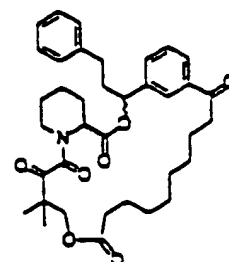
Scheme 6

Table 1
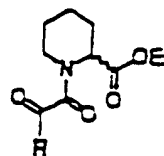
	Example/Compound No.	Structure R
5	35	
	36	
	37	
	38	
	39	
10	40	
	41	
	42	
	43	
	44	
15	45	
	46	
	47	
	48	
	49	
20	50	

57
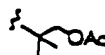
Table 2

Example/Compound No.	Structure
51	
52	
53	
54	
55	

5

58
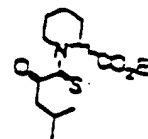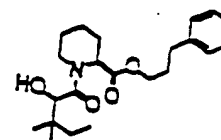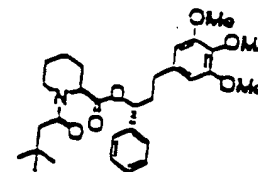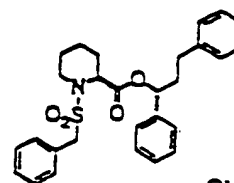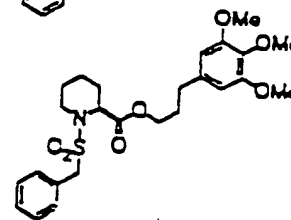
Scheme 7

Table 1
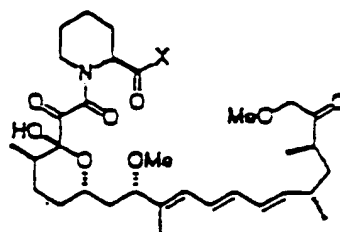
56	x =	OH
57	x =	OMe
58	x =	Oi Pr
59	x =	OBn
60	x =	OCH MePh
61	x =	OCH₂CHCHPh
62	x =	OCH₂CH₂CH₂(3,4-OMe₂)Ph
63	x =	NHBn
64	x =	NHCH₂CH₂CH₂Ph

5

10

Table 2

65	R = Me
66	R = Bn

SUBSTITUTE SHEET (RULE 26)

Table 3

67

68

60
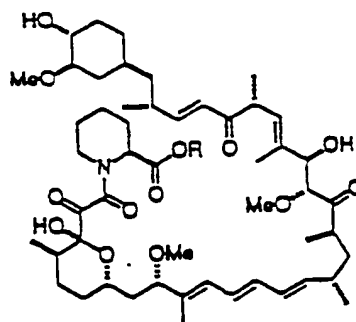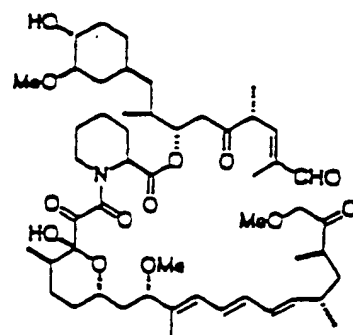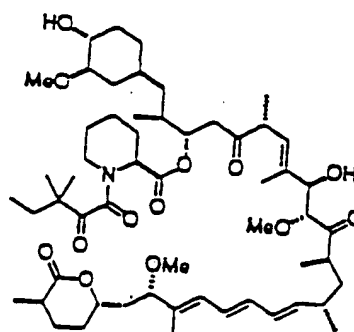
Scheme 8
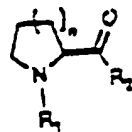
Table 1

Example/Compound No.	Structure
69	n = 2, R_1 = R_2 = Phe-o-tert-butyl
70	n = 2, R_1 = R_2 = Phe-o-tert-butyl
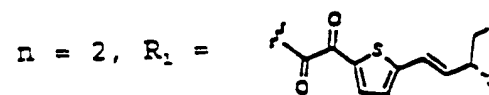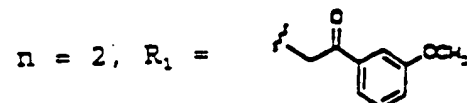
Table 2
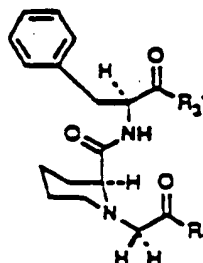
71 R_1=m-OCH$_3$Ph; R_3^1=Val-o-tert-butyl
72 R_1=m-OCH$_3$Ph; R_3^1=Leu-o-tert-butyl
73 R_1=m-OCH$_3$Ph; R_3^1=Ileu-o-tert-butyl
74 R_1=m-OCH$_3$Ph; R_3^1=hexahydro-Phe-o-ter: butyl
75 R_1=m-OCH$_3$Ph; R_3^1=allylalanine-o-tert butyl
76 R_1=B-naphthyl; R_3^1=Val-o-tert-butyl

61

Table 3
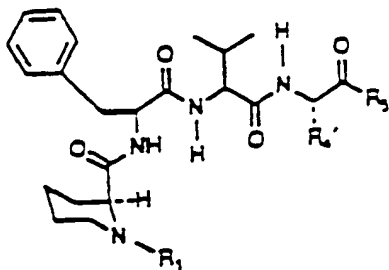
Example/Compound No.	Structure
77	R_1 = $CH_2(CO)$-m-OCH_3Ph R_4 = CH_2Ph R_5 = OCH_3
78	R_1 = $CH_2(CO)$-3-naphthyl R_4 = CH_2Ph R_5 = OCH_3

5

62

Table 4
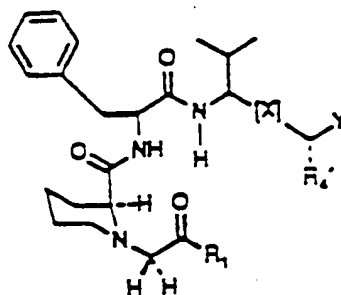
Example/Compound No.	Structure

79

R_1 = m-OCH$_3$Ph
X = trans-CH=CH
R_4^1 = H
Y = OC(o)Ph

80

R_1 = m-OCH$_3$Ph
X = trans-CH=CH
R_4^1 = H
Y = OC(o)CF$_3$

81

R_1 = m-OCH$_3$Ph
X = trans-CH=CHI
R_4^1 = -
Y = -

82

R_1 = m-OCH$_3$Ph
X = trans-CH=CH
R_4^1 = H
Y = OCH$_2$CH=CH$_2$

83

R_1 = m-OCH$_3$Ph
X = C=O
R_4^1 = H
Y = Ph

SUBSTITUTE SHEET (RULE 26)
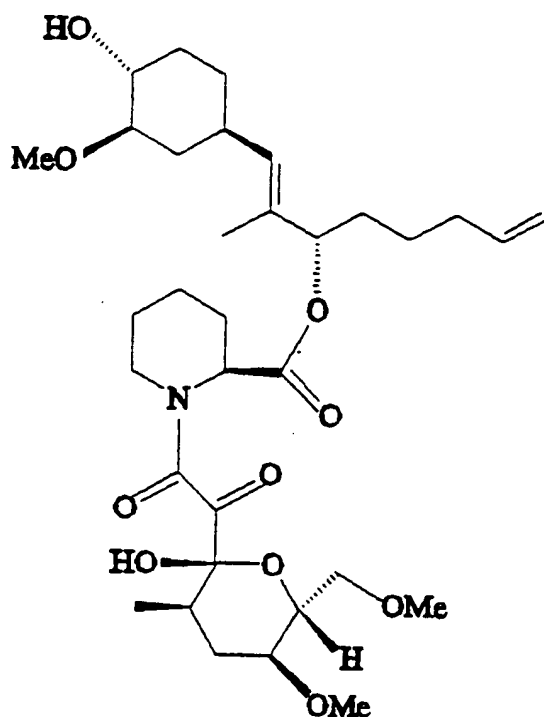
Scheme 9
     Table 1
        85

Table 2
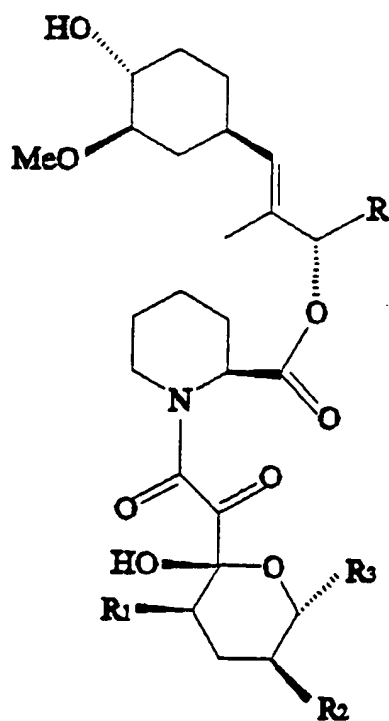
86
87
88

R₁=H, R₂=OMeR₃=CH₂OMe
R₁=H, R₂=R₃=H
R₁=Me, R₂=R₃=H

64

Scheme 10
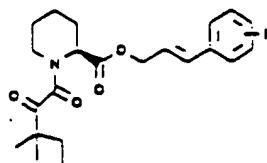
Table 1

	Example	R =
5	90	3,4-dichloro
	91	3,4,5-trimethoxy
	92	H
	93	3-(2,5-Dimethoxy)-phenylpropyl
10	94	3-(3,4-Methylene-dioxy)phenylpropyl

Table 2
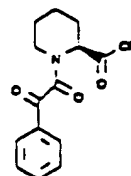
	Example	R =
15	95	4-(p-Methoxy)-butyl
	96	3-Phenylpropyl
	97	3-(3-Pyridyl)-propyl

65

Table 3
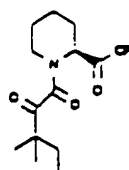
	Example	R =
	98	3-(3-Pyridyl)-propyl
	99	1,7-Diphenyl-4-heptyl
5	100	4-(4-Methoxy)butyl
	101	1-Phenyl-6-(4-methoxy-phenyl)-4-hexyl
	102	3-(2,5-Dimethoxy)phenyl-propyl
10	103	3-(3,4-Methylenedioxy)-phenylpropyl
	104	1,5-Diphenylpentyl

Table 4
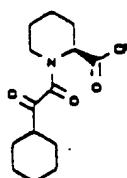
15	Example	R =
	105	4-(4-Methoxy)butyl
	106	3-Cyclohexylpropyl
	107	3-Phenylpropyl

Table 5
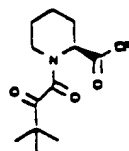
20		
	Example	R =
	108	3-Cyclohexylpropyl
	109	3-Phenylpropyl
	110	4-(4-Methoxy)butyl
25	111	1,7-Diphenyl-4-heptyl

NEUROTROPHIC EFFECTS OF ROTAMASE INHIBITORS

Table I lists a number of the claimed examples
together with their potencies to induce trophic effects
in cultured sensory neurons, as described above.
Figures 19 and 20 show photomicrographs of Example 111
and Example 17 promoting neurite outgrowth in the dorsal
root ganglion cultures.

Table I

In Vitro Potencies of Test Examples

Example	Rotamase Inhibition K_i, nM	Neutrophic ED_{50} Chick DRGs, nM
6	140	25
9	13	0.030
11	170	1
12	250	300
13	25	80
15	17	0.30
19	12	0.017
36	>10,000	>10,000
41	1300	5000
50	>10,000	>10,000
90	1800	2500
91	28	200
92	39	90
93	75	35
94	70	8
95	165	5-10
96	740	10-20
97	725	150
98	130	75
99	30	5
100	60	43
101	15	0.17
102	12	2.5
103	120	3
104	20	.016
105	103	6
106	760	1
107	210	0.82
108	32	0.29
109	2	0.08
110	24	0.002
111	5	0.08

ACTIVITY OF EXAMPLE COMPOUNDS IN IN VIVO MODEL OF
NERVE REGENERATION

Sciatic Nerve Axotomy

    Six-week old male Sprague-Dawley rats were
anesthetized, and the sciatic nerve exposed and crushed,
at the level of the hip, by forceps. Test compounds or
vehicle were administered subcutaneously just prior to
the lesion and daily for the following 18 days. Sections
of the sciatic nerve were stained with Holmes silver
stain to quantify the number of axons, and Luxol fast
blue to quantify the level of myelination. Eighteen days
after lesion, there was a significant decrease in the
number of axons (50% decrease as compared to non-lesioned
control) and degree of myelination (90% decrease as
compared to non-lesioned control) in animal treated with
vehicle.

    Administration of Example 12 (30 mg/kg s.c.), or
Example 13 (mg/kg s.c.) just prior to the lesion and
daily for 18 days following the lesion, resulted in
significant regeneration of both axon number (25% and 5%
decrease, respectively, as compared to non-lesioned
control) and the degree of myelination (65 % and 50%
decrease, respectively, as compared to control) as
compared to vehicle treated animals. The significant
efficacy of Examples 12 and 13 are consistent with their
potent activity in inhibiting rotamase activity and
stimulating neurite outgrowth in chick DRGs, and their
relative potencies in vivo parallel their in vitro
potencies (Table I). These results are shown in Figure
21. "Sham" denotes control animals that received vehicle
but were not lesioned; "Vehicle" denotes animals that
were lesioned and received only vehicle (i.e., no drug).
Example 12 and Example 13 showed a striking similarity to
the sham treated animals, demonstrating the powerful
neuroregenerative effects of these compounds in vivo.
These data are quantitated in Table II.

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Table II

Treatment	Axon Number (% Control)	Myelin Level
Sham	100	100
Lesion:		
+ Vehicle (s.c.)	50	10
+ Example 12 (30 mg/kg s.c.)	75	35
+ Example 13 (30 mg/kg s.c.)	100	50

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The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.

15

What is claimed is:

1.   A method of treating a neurological disorder in an
animal, comprising:
      administering to an animal an effective amount of a
5 pipecolic acid derivative having an affinity for FKBP-type
immunophilins to stimulate growth of damaged peripheral nerves
or to promote neuronal regeneration, wherein the FKBP-type
immunophilin exhibits rotamase activity and the pipecolic
acid derivative inhibits said rotamase activity of the
10 immunophilin.
2.   The method of claim 1, wherein the neurological
disorder is selected from the group consisting of peripheral
neuropathies cause by physical injury or disease state,
physical damage to the brain, physical damage to the spinal
15 cord, stroke associated with brain damage, and neurological
disorders relating to neurodegeneration.
3.   The method of claim 2, wherein the neurological
disorder is selected from the group consisting of
Alzheimer's Disease, Parkinson's Disease, and amyotrophic
20 lateral sclerosis.
4.   The method of claim 1, wherein the pipecolic acid
derivative compound is immunosuppressive or non-
immunosuppressive.
5.   The method of claim 1, wherein the pipecolic acid
25 derivative is Way-124,666.
6.   The method of claim 1, wherein the pipecolic acid
derivative is rapamycin.
7.   The method of claim 1, wherein the pipecolic acid
derivative is FK506.
30 8.   The method of claim 1, wherein the pipecolic acid
derivative is Rap-Pa.

9.   The method of claim 1, wherein the pipecolic acid derivative is SLB-506.

10.   The method of claim 1, wherein the pipecolic acid derivative is selected from the group consisting of compounds
5 3-84, and 86-88.

11.   A method of treating a neurological disorder in an animal, comprising:

administering to an animal an effective amount of a pipecolic acid derivative having an affinity for FKBP-type
10 immunophilins in combination with an effective amount of a neurotrophic .factor selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, cilial neurotrophic factor, and neurotropin-3, to stimulate growth of damaged peripheral
15 nerves or to promote neuronal regeneration, wherein the FKBP-type immunophilin exhibits rotamase activity and the pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

12.   The method of claim 11, wherein the neurological
20 disorder is selected from the group consisting of peripheral neuropathies cause by physical injury or disease state, physical damage to the brain, physical damage to the spinal cord, stroke associated with brain damage, and neurological disorders relating to neurodegeneration.
25 13.   The method of claim 12, wherein the neurological disorder is selected from the group consisting of Alzheimer's Disease, Parkinson's Disease, and amyotrophic lateral sclerosis.

14.   The method of claim 11, wherein the pipecolic acid
30 derivative compound is immunosuppressive or non-immunosuppressive.

15.   The method of claim 11, wherein the pipecolic acid derivative is Way-124,666.

16.   The method of claim 11, wherein the pipecolic acid derivative is rapamycin.

5 17.   The method of claim 11, wherein the pipecolic acid derivative is FK506.

18.   The method of claim 11, wherein the pipecolic acid derivative is Rap-Pa.

19.   The method of claim 11, wherein the pipecolic acid 10 derivative is SLB-506.

20.   The method of claim 11, wherein the pipecolic acid derivative is selected from the group consisting of compounds 3-84, and 86-88.

21.   A method of stimulating growth of damaged peripheral 15 nerves, comprising;

administering to damaged peripheral nerves an effective amount of a pipecolic acid derivative compound having an affinity for FKBP-type immunophilins to stimulate or promote growth of the damaged peripheral nerves, wherein the FKBP- 20 type immunophilins exhibit rotamase activity and the pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

22.   The method of claim 21, further comprising administering a neurotrophic factor to stimulate or promote 25 growth of the damaged peripheral nerves selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, cilial neurotrophic factor, and neurotropin-3.

23.   The method of claim 21, wherein the pipecolic acid 30 derivative is immunosuppressive or non-immunosuppressive.

24. The method of claim 21, wherein the pipecolic acid derivative is Way-124,666

25. The method of claim 21, wherein the pipecolic acid derivative is rapamycin.

26. The method of claim 21, wherein the pipecolic acid derivative is FK506.

27. The method of claim 21, wherein the pipecolic acid derivative is Rap-Pa.

28. The method of claim 21, wherein the pipecolic acid derivative is SLB-506.

29. The method of claim 21, wherein the pipecolic acid derivative is selected from the group consisting of compounds 3-84, and 86-88.

30. A method of stimulating growth of damaged peripheral nerves, comprising:

administering to damaged peripheral nerves an effective amount of a pipecolic acid derivative compound having an affinity for FKBP-type immunophilins to stimulate growth of damaged peripheral nerves, wherein the FKBP-type immunophilin exhibit rotamase activity and the pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

31. The method of claim 30, further comprising administering an effective amount of a neurotrophic factor selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, cilial neurotrophic factor, and neurotropin-3 to stimulate the growth of damaged peripheral nerves.

32. The method of claim 30, wherein the pipecolic acid derivative c mpound is immunosuppressive or non-immunosuppressive.

33. The method of claim 30, wherein the pipecolic acid derivative is Way-124,666

34. The method of claim 30, wherein the pipecolic acid derivative is rapamycin.

35. The method of claim 30, wherein the pipecolic acid derivative is FK506.

36. The method of claim 30, wherein the pipecolic acid derivative is Rap-Pa.

37. The method of claim 30, wherein the pipecolic acid derivative is SLB-506.

38. The method of claim 30, wherein the pipecolic acid derivative is selected from the group consisting of compounds 3-84, and 86-88.

39. A method for promoting neuronal regeneration and growth in animals, comprising:

administering to an animal an effective amount of a pipecolic acid derivative compound having an affinity for FKBP-type immunophilins to promote neuronal regeneration, wherein the FKBP-type immunophilins exhibit rotamase activity and the pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

40. The method of claim 39, further comprising administering an effective amount of a neurotrophic factor to promote neuronal regeneration selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, and neurotropin-3.

41. The method of claim 39, wherein the pipecolic acid derivative compound is immunosuppressive or non-immunosuppressive.

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42.   The method of claim 39, wherein the pipecolic acid derivative is Way-124,666.

43.   The method of claim 39, wherein the pipecolic acid derivative is rapamycin.

44.   The method of claim 39, wherein the pipecolic acid derivative is FK506.

45.   The method of claim 39, wherein the pipecolic acid derivative is Rap-Pa.

46.   The method of claim 39, wherein the pipecolic acid derivative is SLB-506.

47.   The method of claim 39, wherein the pipecolic acid derivative is selected from the group consisting of compounds 3-84, and 86-88.

48.   A method for preventing neurodegeneration in an animal, comprising:

        administering to an animal an effective amount of a pipecolic acid derivative having an affinity for FKBP-type immunophilins to prevent neurodegeneration, wherein the FKBP-type immunophilin exhibits rotamase activity and the pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

49.   The method of claim 48, further comprising administering an effective amount of a neurotrophic factor to prevent neurodegeneration selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, cilial neurotrophic factor, and neurotropin-3.

50.   The method of claim 48, wherein the pipecolic acid derivative compound is immunosuppressive or non-immunosuppressive.

51. The method of claim 48, wherein the pipecolic acid derivative is Way-124,666.

52. The method of claim 48, wherein the pipecolic acid derivative is rapamycin.

5   53. The method of claim 48, wherein the pipecolic acid derivative is FK506.

54. The method of claim 48, wherein the pipecolic acid derivative is Rap-Pa.

55. The method of claim 48, wherein the pipecolic acid

10   derivative is SLB-506.

56. The method of claim 48, wherein the pipecolic acid derivative is selected from the group consisting of compounds 3-84, and 86-88.

AMENDED CLAIMS
[received by the International Bureau on 28 November 1996 (28.11.96);
original claims 4,6,7,14,16, 17,23,25,26,32,34,35,41,43,44,50,52 and 53
cancelled; original claims 1,5, 8-11,15,18-21,24,27-30,33,36-39,42,45-48,
51 and 54-56 amended; remaining claims unchanged (12 pages)]

1.   A method of treating a neurological disorder in an

animal, comprising:

        administering to an animal an effective amount of a

        non-immunosuppressive pipecolic acid derivative having

        an affinity for FKBP-type immunophilins to stimulate

        growth of damaged peripheral nerves or to promote

        neuronal regeneration, wherein the FKBP-type

        immunophilin exhibits rotamase activity and the

        pipecolic acid derivative inhibits said rotamase

        activity of the immunophilin.

2.   The method of claim 1, wherein the neurological

disorder is selected from the group consisting of

peripheral neuropathies cause by physical injury or disease

state, physical damage to the brain, physical damage to the

spinal cord, stroke associated with brain damage, and

neurological disorders relating to neurodegeneration.

3.   The method of claim 2, wherein the neurological

disorder is selected from the group consisting of

Alzheimer's Disease, Parkinson's Disease, and amyotrophic

lateral sclerosis.

5.   The method of claim 1, wherein the non-

immunosuppressive pipecolic acid derivative is Way-124,666.

8. The method of claim 1, wherein the non-immunosuppressive pipecolic acid derivative is Rap-Pa.

9. The method of claim 1, wherein the non-immunosuppressive pipecolic acid derivative is SLB-506.

10. The method of claim 1, wherein the non-immunosuppressive pipecolic acid derivative is selected from the group consisting of compounds 3-84, 86-88 and 90-111.

11. The method of claim 1, wherein the method provides at least 75% regeneration of axon number and at least 35% recovery of myelination.

12. The method of claim 1, wherein the method provides at least 75% regeneration of axon number and at least 50% recovery of myelination.

13. The method of claim 1, wherein rotamase inhibition is achieved using no greater than a 1800 nM concentration of the compound.

14. The method of claim 1, wherein rotamase inhibition is achieved using no greater than a 210 nM concentration of the compound.

15. The method of claim 1, wherein rotamase inhibition is

achieved using no greater than a 103 nM concentration of the compound.

16. The method of claim 1, wherein neurotrophic ED_{50} is achieved using no greater than a 200 nM concentration of the compound.

17. The method of claim 1, wherein neurotrophic ED_{50} is achieved using no greater than a 35 nM concentration of the compound.

18. The method of claim 1, wherein neurotrophic ED_{50} is achieved using no greater than a 1 nM concentration of the compound.

19. A method of treating a neurological disorder in an animal, comprising:

administering to an animal an effective amount of a non-immunosuppressive pipecolic acid derivative having an affinity for FKBP-type immunophilins in combination with an effective amount of a neurotrophic factor selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, cilial neurotrophic factor, and neurotropin-3, to stimulate growth of damaged peripheral nerves or to promote neuronal regeneration, wherein the FKBP-type immunophilin exhibits rotamase activity and the non-immunosuppressive pipecolic acid

derivative inhibits said rotamase activity of the
immunophilin.

20. The method of claim 19, wherein the neurological
disorder is selected from the group consisting of
peripheral neuropathies cause by physical injury or disease
state, physical damage to the brain, physical damage to the
spinal cord, stroke associated with brain damage, and
neurological disorders relating to neurodegeneration.

21. The method of claim 19, wherein the neurological
disorder is selected from the group consisting of
Alzheimer's Disease, Parkinson's Disease, and amyotrophic
lateral sclerosis.

22. The method of claim 19, wherein the non-
immunosuppressive pipecolic acid derivative is Way-124,666.

23. The method of claim 19, wherein the non-
immunosuppressive pipecolic acid derivative is Rap-Pa.

24. The method of claim 19, wherein the non-
immunosuppressive pipecolic acid derivative is SLB-506.

25. The method of claim 19, wherein the non-
immunosuppressive pipecolic acid derivative is selected
from the group consisting of compounds 3-84, 86-88 and 90-
111.

AMENDED SHEET (ARTICLE 19)

26.   A method of stimulating growth of damaged peripheral
nerves, comprising;

          administering to damaged peripheral nerves an

          effective amount of a non-immunosuppressive pipecolic

          acid derivative compound having an affinity for FKBP-

          type immunophilins to stimulate or promote growth of

          the damaged peripheral nerves, wherein the FKBP-type

          immunophilins exhibit rotamase activity and the non-

          immunosuppressive pipecolic acid derivative inhibits

          said rotamase activity of the immunophilin.

27.   The method of claim 26, further comprising
administering a neurotrophic factor to stimulate or promote
growth of the damaged peripheral nerves selected from the
group consisting of neurotrophic growth factor, brain
derived growth factor, glial derived growth factor, cilial
neurotrophic factor, and neurotropin-3.

28.   The method of claim 26, wherein the non-
immunosuppressive pipecolic acid derivative is Way-124,666

29.   The method of claim 26, wherein the non-
immunosuppressive pipecolic acid derivative is Rap-Pa.

30.   The method of claim 26, wherein the non-
immunosuppressive pipecolic acid derivative is SLB-506.

31.   The method of claim 26, wherein the non-

immunosuppressive pipecolic acid derivative is selected from the group consisting of compounds 3-84, 86-88 and 90-111.

32.   The method of claim 26, wherein the method provides at least 75% regeneration of axon number and at least 35% recovery of myelination.

33.   The method of claim 26, wherein the method provides at least 75% regeneration of axon number and at least 50% recovery of myelination.

34.   The method of claim 26, wherein rotamase inhibition is achieved using no greater than a 1800 nM concentration of the compound.

35.   The method of claim 26, wherein rotamase inhibition is achieved using no greater than a 210 nM concentration of the compound.

36.   The method of claim 26, wherein rotamase inhibition is achieved using no greater than a 103 nM concentration of the compound.

37.   The method of claim 26, wherein neurotrophic ED_{50} is achieved using no greater than a 200 nM concentration of the compound.

38. The method of claim 26, wherein neurotrophic ED_{50} is achieved using no greater than a 35 nM concentration of the compound.

39. The method of claim 26, wherein neurotrophic ED_{50} is achieved using no greater than a 1 nM concentration of the compound.

40. A method of stimulating growth of damaged peripheral nerves, comprising:

administering to damaged peripheral nerves an effective amount of a non-immunosuppressive pipecolic acid derivative compound having an affinity for FKBP-type immunophilins to stimulate growth of damaged peripheral nerves, wherein the FKBP-type immunophilin exhibit rotamase activity and the non-immunosuppressive pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

41. The method of claim 40, further comprising administering an effective amount of a neurotrophic factor selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, cilial neurotrophic factor, and neurotropin-3 to stimulate the growth of damaged peripheral nerves.

42. The method of claim 40, wherein the non-immunosuppressive pipecolic acid derivative is Way-124,666.

43.   The method of claim 40, wherein the non-immunosuppressive pipecolic acid derivative is Rap-Pa.

44.   The method of claim 40, wherein the non-immunosuppressive pipecolic acid derivative is SLB-506.

45.   The method of claim 40, wherein the non-immunosuppressive pipecolic acid derivative is selected from the group consisting of compounds 3-84, 86-88 and 90-111.

46.   A method for promoting neuronal regeneration and growth in animals, comprising:

administering to an animal an effective amount of a non-immunosuppressive pipecolic acid derivative compound having an affinity for FKBP-type immunophilins to promote neuronal regeneration, wherein the FKBP-type immunophilins exhibit rotamase activity and the non-immunosuppressive pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

47.   The method of claim 46, further comprising administering an effective amount of a neurotrophic factor to promote neuronal regeneration selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, and neurotropin-3.

48.   The method of claim 46, wherein the non-immunosuppressive pipecolic acid derivative is Way-124,666.

49.   The method of claim 46, wherein the non-immunosuppressive pipecolic acid derivative is Rap-Pa.

50.   The method of claim 46, wherein the non-immunosuppressive pipecolic acid derivative is SLB-506.

51.   The method of claim 46, wherein the non-immunosuppressive pipecolic acid derivative is selected from the group consisting of compounds 3-84, 86-88 and 90-111.

52.   The method of claim 46, wherein the method provides at least 75% regeneration of axon number and at least 35% recovery of myelination.

53.   The method of claim 46, wherein the method provides at least 75% regeneration of axon number and at least 50% recovery of myelination.

54.   The method of claim 46, wherein rotamase inhibition is achieved using no greater than a 1800 nM concentration of the compound.

55.   The method of claim 46, wherein rotamase inhibition is achieved using no greater than a 210 nM concentration of

the compound.

56. The method of claim 46, wherein rotamase inhibition is achieved using no greater than a 103 nM concentration of the compound.

57. The method of claim 46, wherein neurotrophic ED_{50} is achieved using no greater than a 200 nM concentration of the compound.

58. The method of claim 46, wherein neurotrophic ED_{50} is achieved using no greater than a 35 nM concentration of the compound.

59. The method of claim 46, wherein neurotrophic ED_{50} is achieved using no greater than a 1 nM concentration of the compound.

60. A method for preventing neurodegeneration in an animal, comprising:

administering to an animal an effective amount of a non-immunosuppressive pipecolic acid derivative having an affinity for FKBP-type immunophilins to prevent neurodegeneration, wherein the FKBP-type immunophilin exhibits rotamase activity and the non-immunosuppressive pipecolic acid derivative inhibits said rotamase activity of the immunophilin.

61. The method of claim 60, further comprising administering an effective amount of a neurotrophic factor to prevent neurodegeneration selected from the group consisting of neurotrophic growth factor, brain derived growth factor, glial derived growth factor, cilial neurotrophic factor, and neurotropin-3.

62. The method of claim 60, wherein the non-immunosuppressive pipecolic acid derivative is Way-124,666.

63. The method of claim 60, wherein the non-immunosuppressive pipecolic acid derivative is Rap-Pa.

64. The method of claim 60, wherein the non-immunosuppressive pipecolic acid derivative is SLB-506.

65. The method of claim 60, wherein the non-immunosuppressive pipecolic acid derivative is selected from the group consisting of compounds 3-84, 86-88 and 90-111.

66. The method of claim 60, wherein the method provides at least 75% regeneration of axon number and at least 35% recovery of myelination.

67. The method of claim 60, wherein the method provides at least 75% regeneration of axon number and at least 50% recovery of myelination.

68. The method of claim 60, wherein rotamase inhibition is achieved using no greater than a 1800 nM concentration of the compound.

69. The method of claim 60, wherein rotamase inhibition is achieved using no greater than a 210 nM concentration of the compound.

70. The method of claim 60, wherein rotamase inhibition is achieved using no greater than a 103 nM concentration of the compound.

71. The method of claim 60, wherein neurotrophic ED_{50} is achieved using no greater than a 200 nM concentration of the compound.

72. The method of claim 60, wherein neurotrophic ED_{50} is achieved using no greater than a 35 nM concentration of the compound.

73. The method of claim 60, wherein neurotrophic ED_{50} is achieved using no greater than a 1 nM concentration of the compound.

FKBP

GAP-43

A

1 DAY

7 DAY

14 DAY

21 DAY

B

FKBP
CONTROL

7 DAY
CALCINEURIN

FIG. I
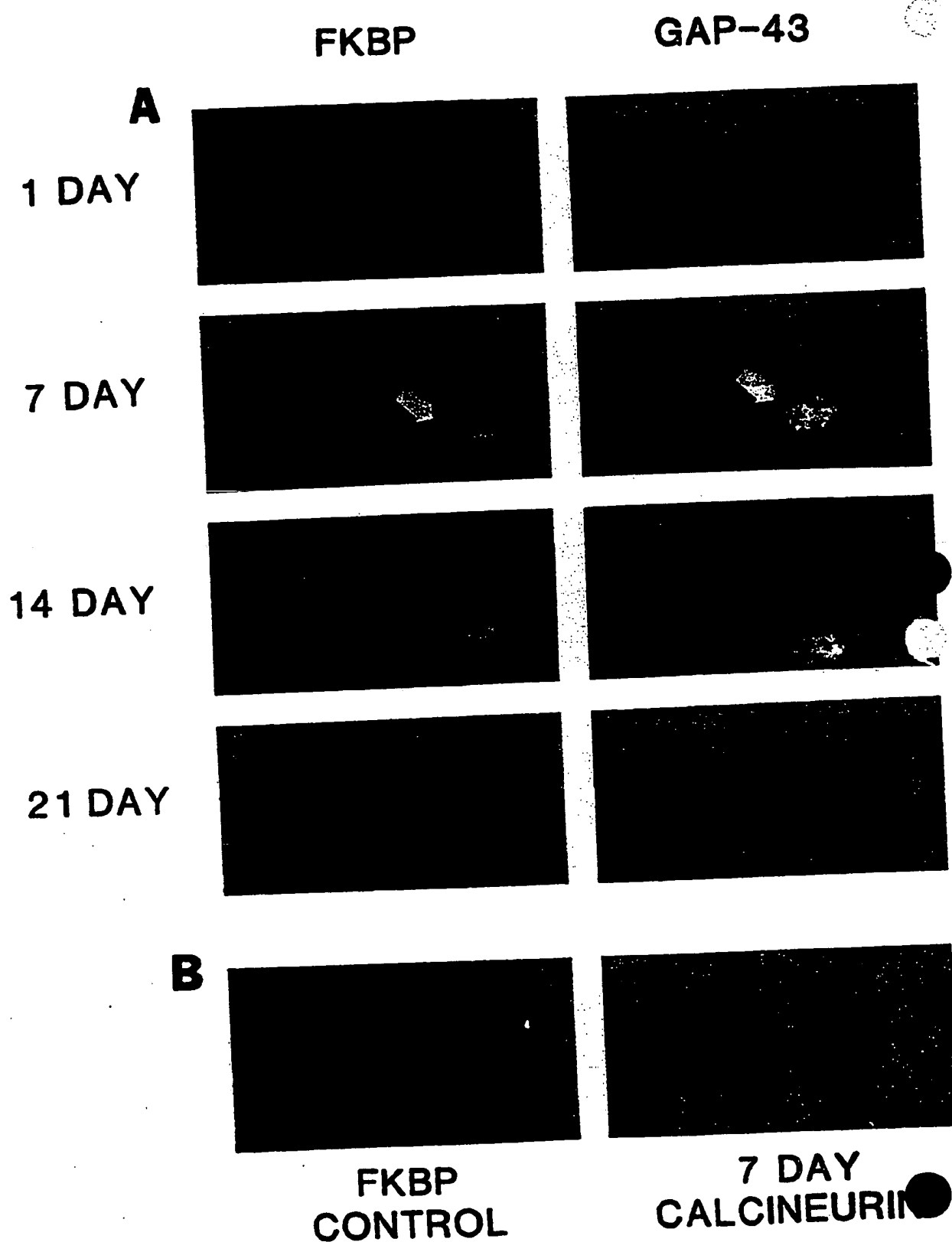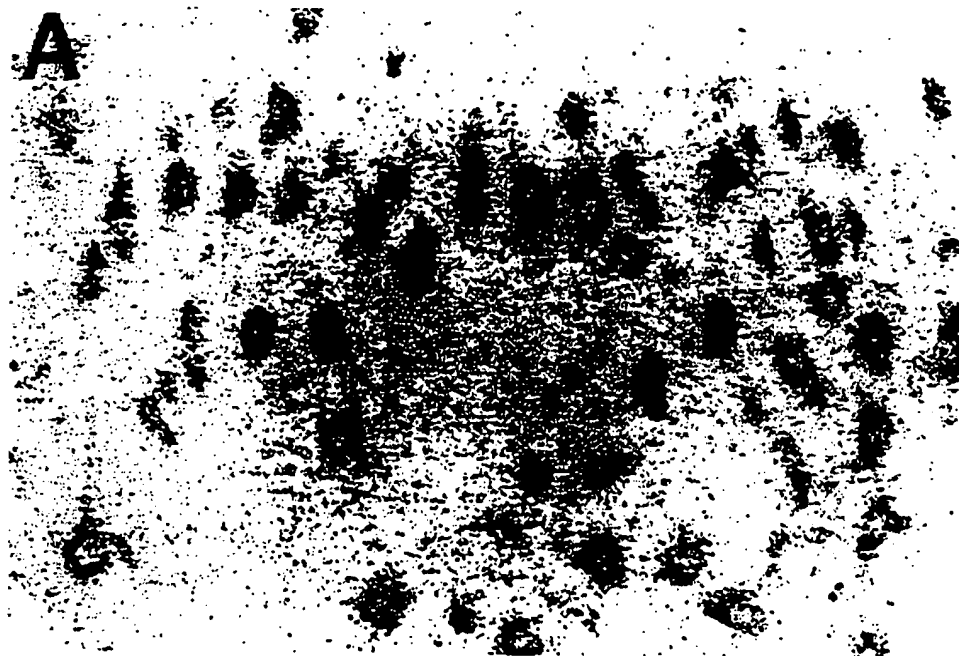
FIG. 2A
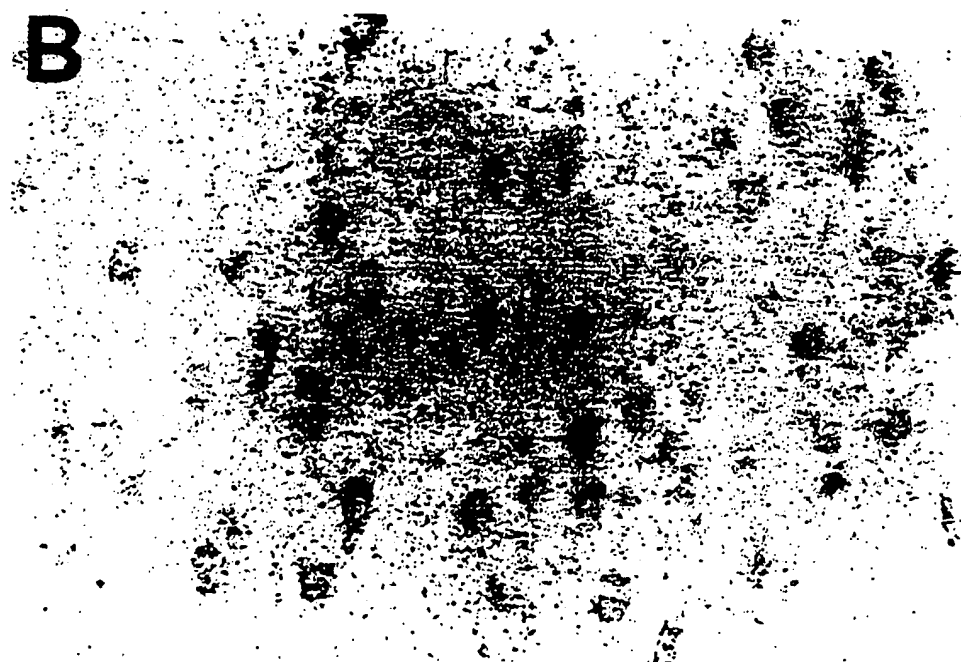
FIG. 2B

FIG. 3A
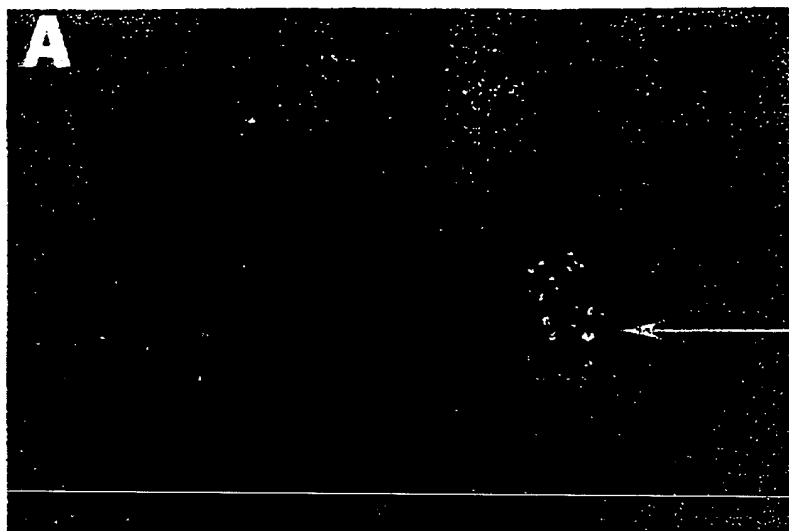
FIG. 3B   FIG. 3C
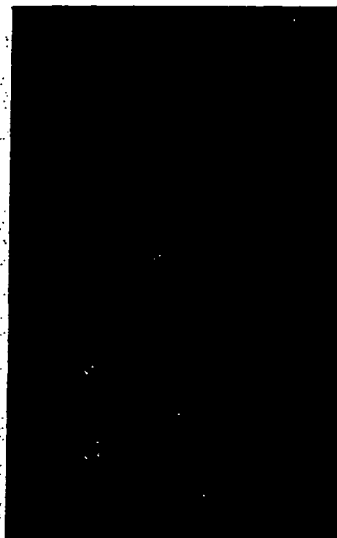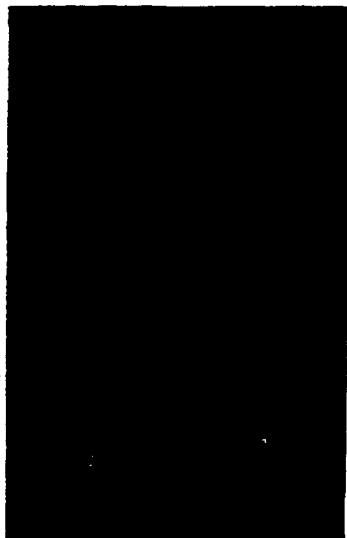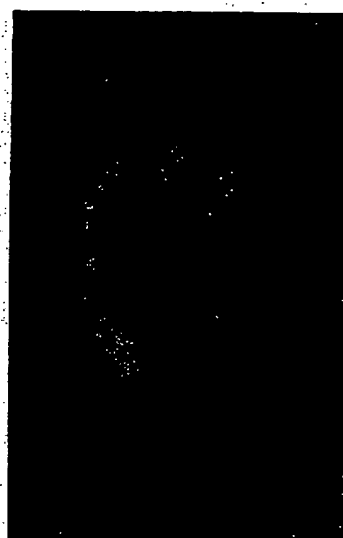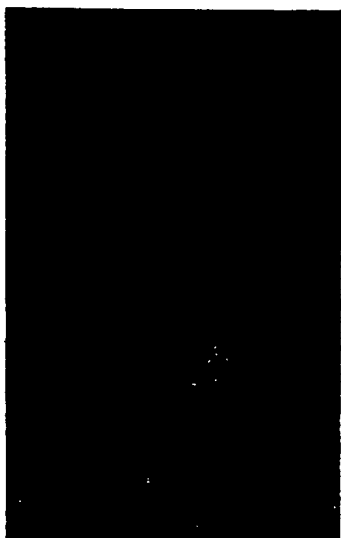
FIG. 4
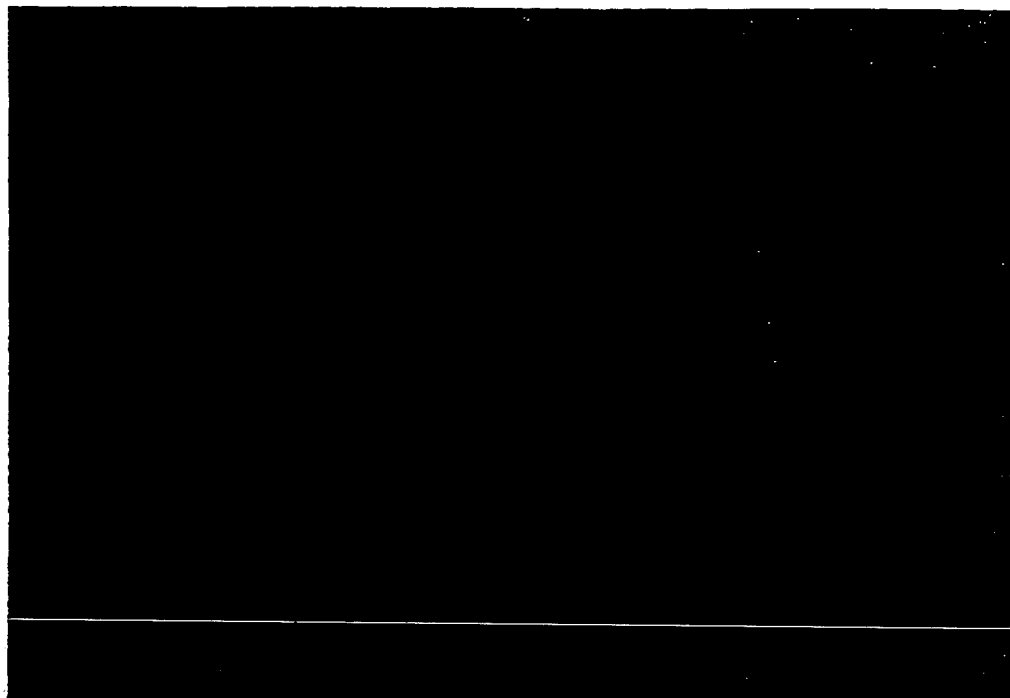
FIG. 5A
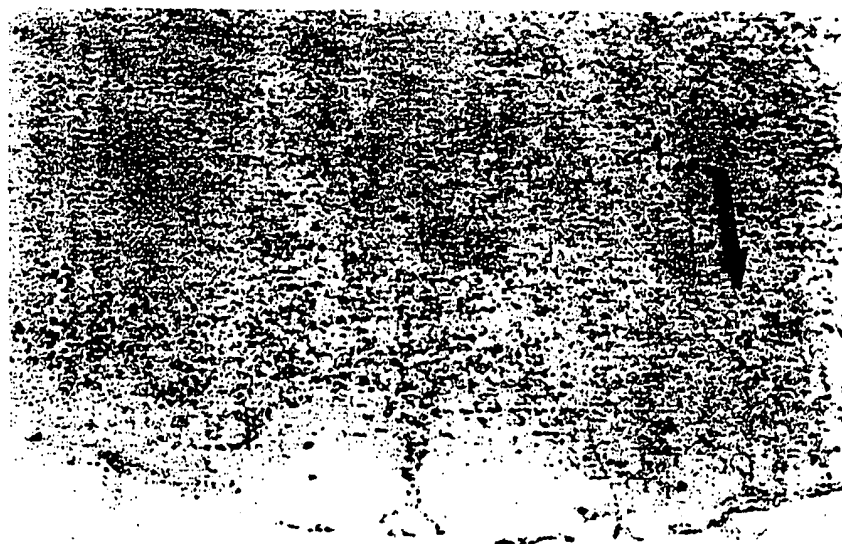
FIG. 5B

FIG. 6
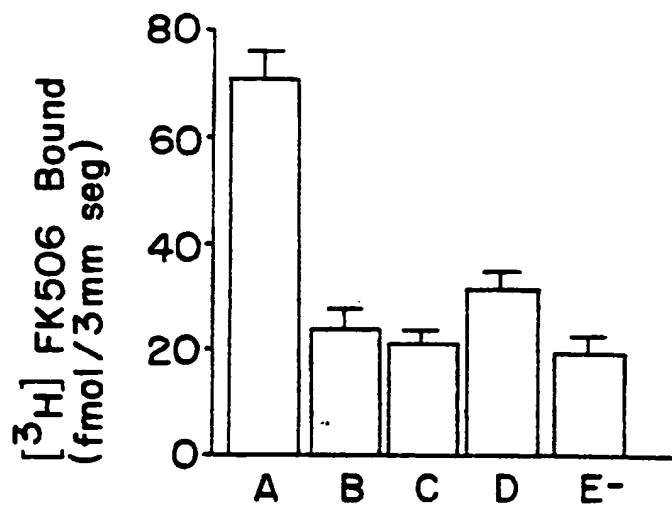
FIG. 8
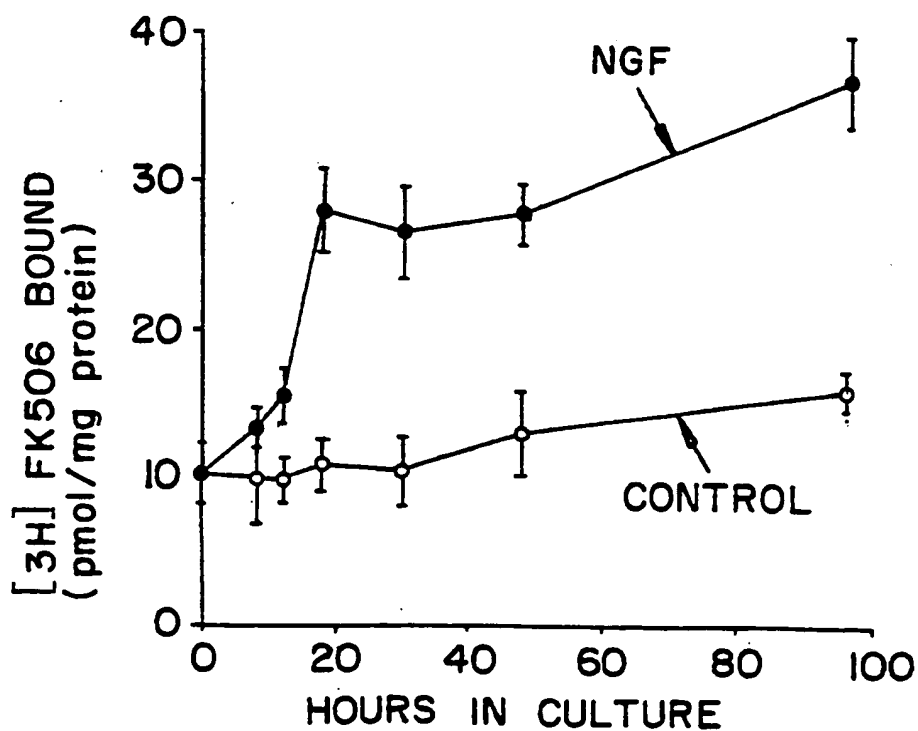
FIG. 7A

FKBP IN SITU

Control

FKBP IN SITU

Crush

FIG. 7B

FIG. 7C

FK-506 AUTORAD

Control
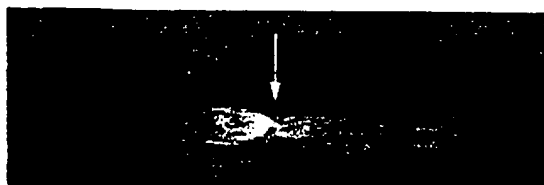
FK-506 AUTORAD
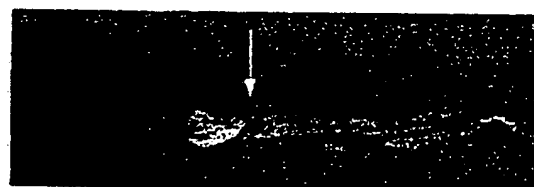
Crush

FIG. 7D

FIG. 9B

FIG. 9D

FIG. 9A

FIG. 9C

FIG.10

FIG.11

SUBSTITUTE SHEET
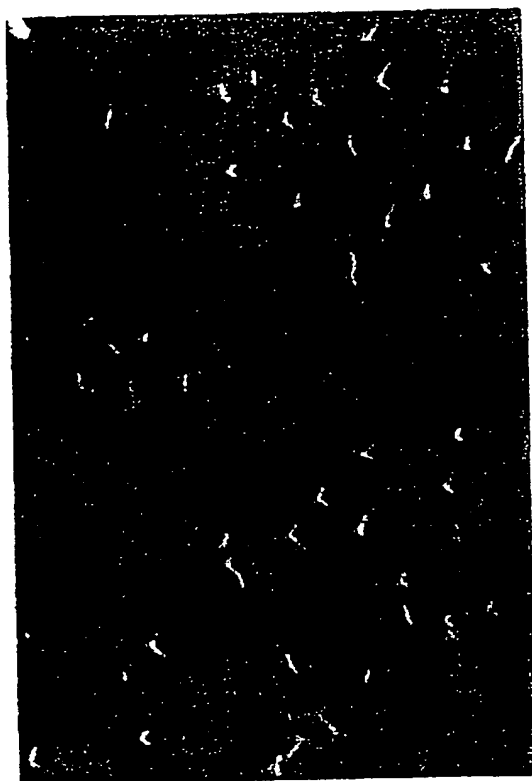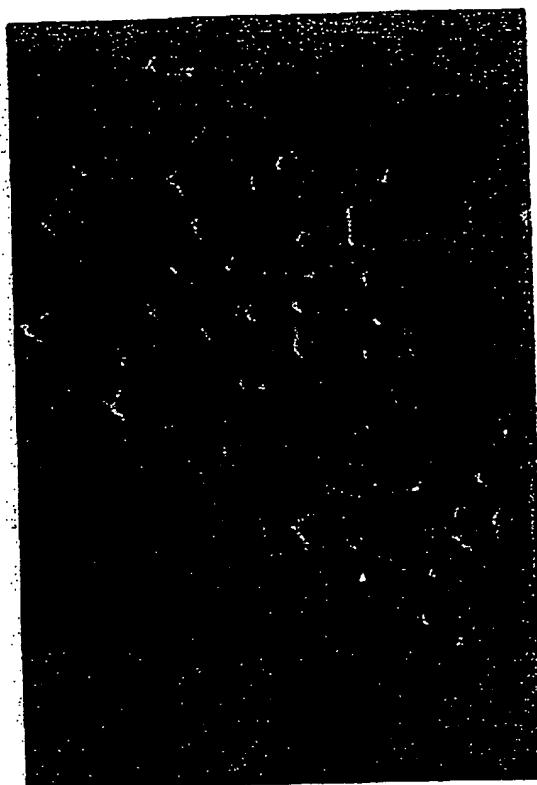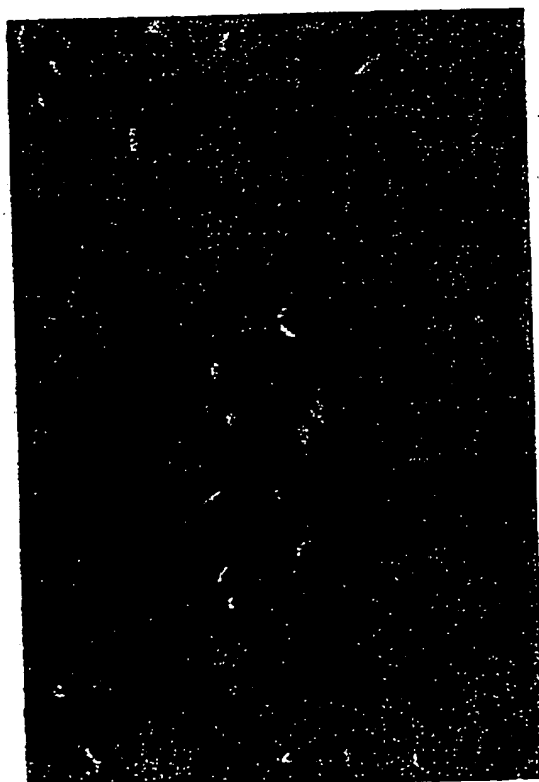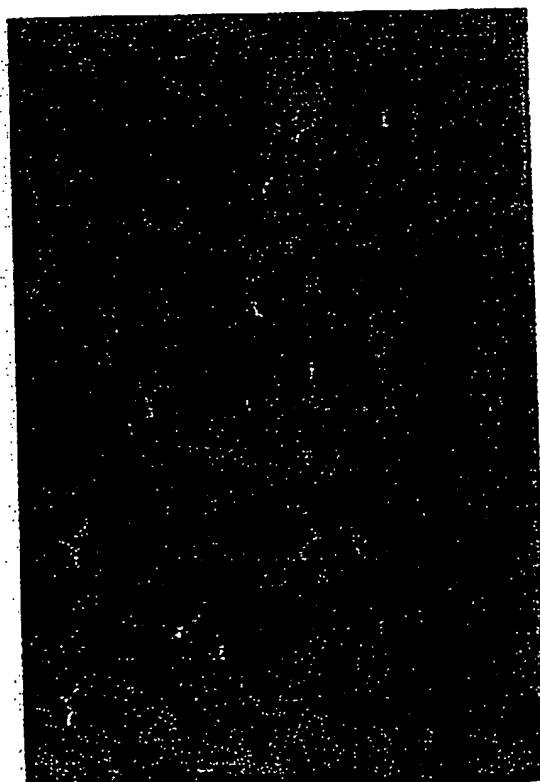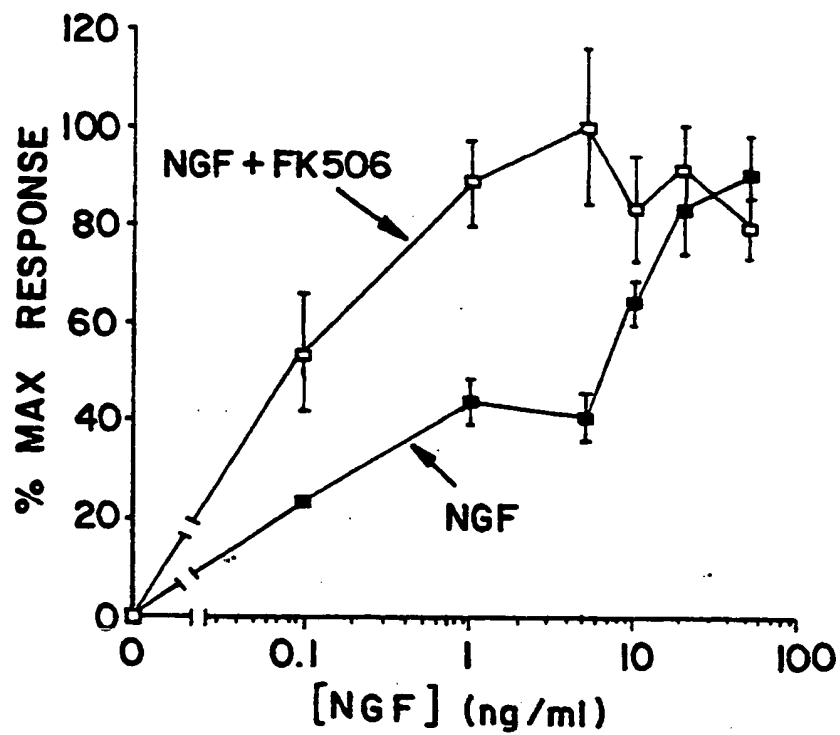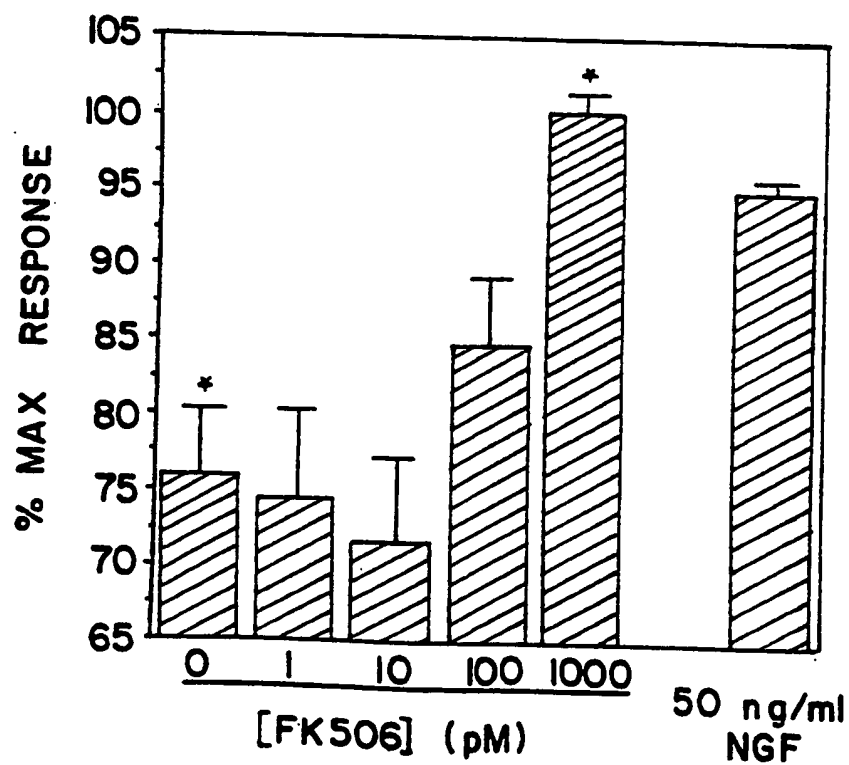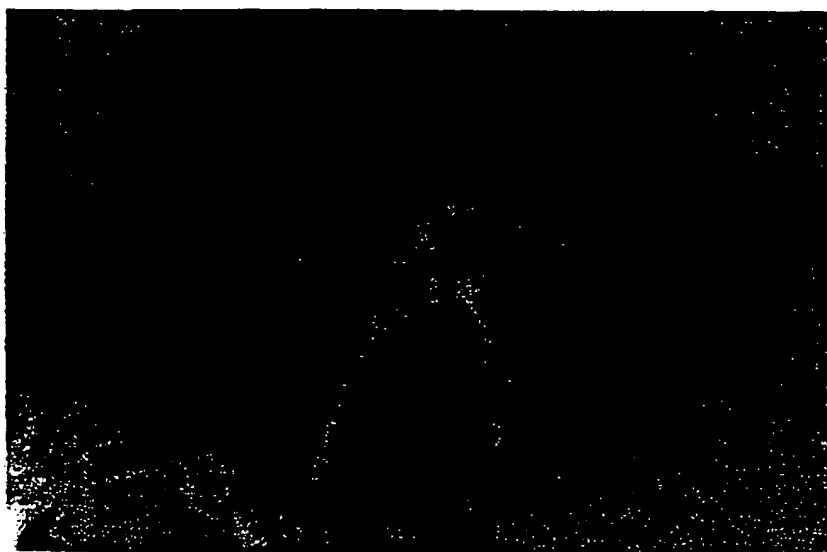
FIG. 12
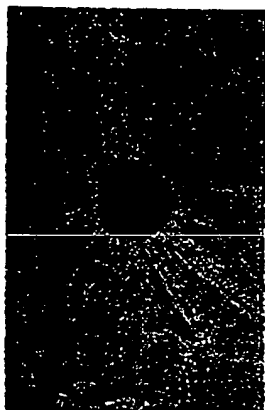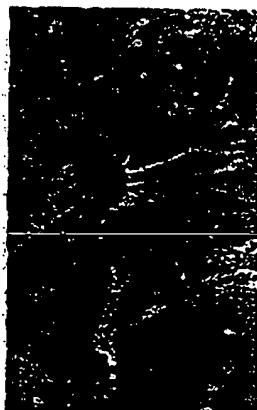
FIG. 13A   FIG. 13B   FIG. 13C

FIG. 13D   FIG. 13E   FIG. 13F

FIG.14

NGF (ng/ml)

Neurite Outgrowth (Cells bearing neurites/ cell number)

■ NGF
□ FK506 100 nM
▨ WAY-124, 466 100 nM
▨ Rapamycin 100 nM

FIG.15

• Rapamycin
○ WAY-124,466
□ FK506
L 0.5 ng/ml NGF
H 50 ng/ml NGF

Neurite extension (Cells bearing
neurites/cell number)

Drug (nM)
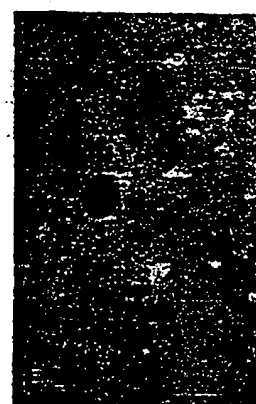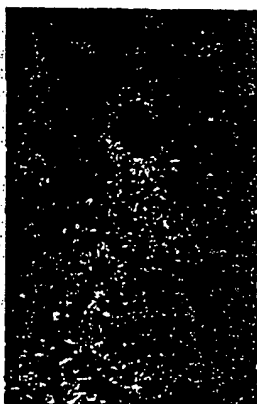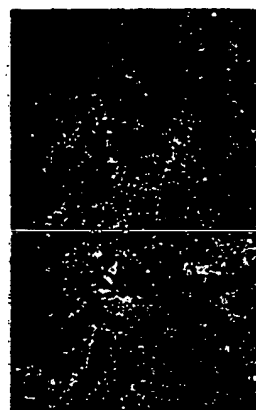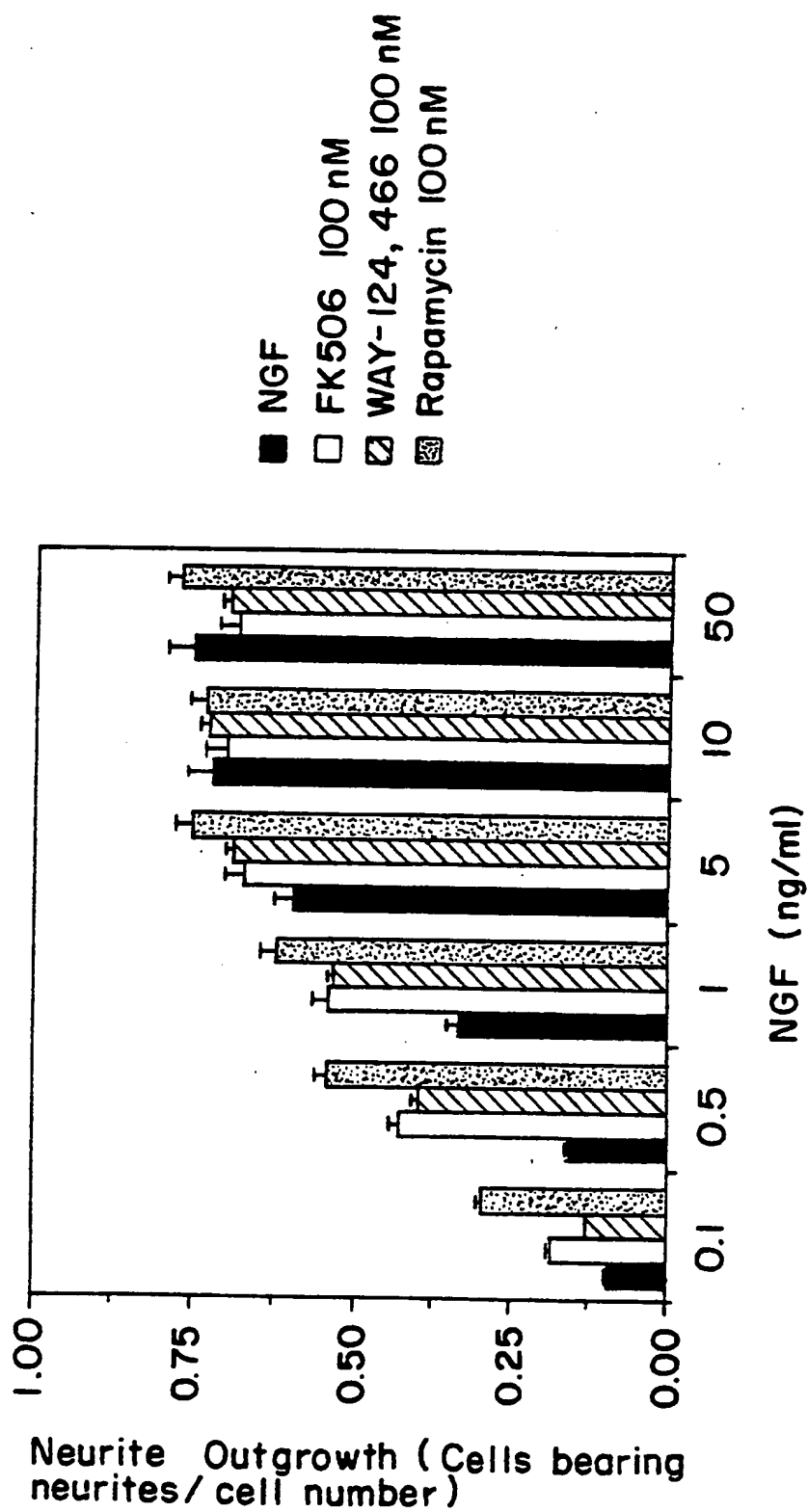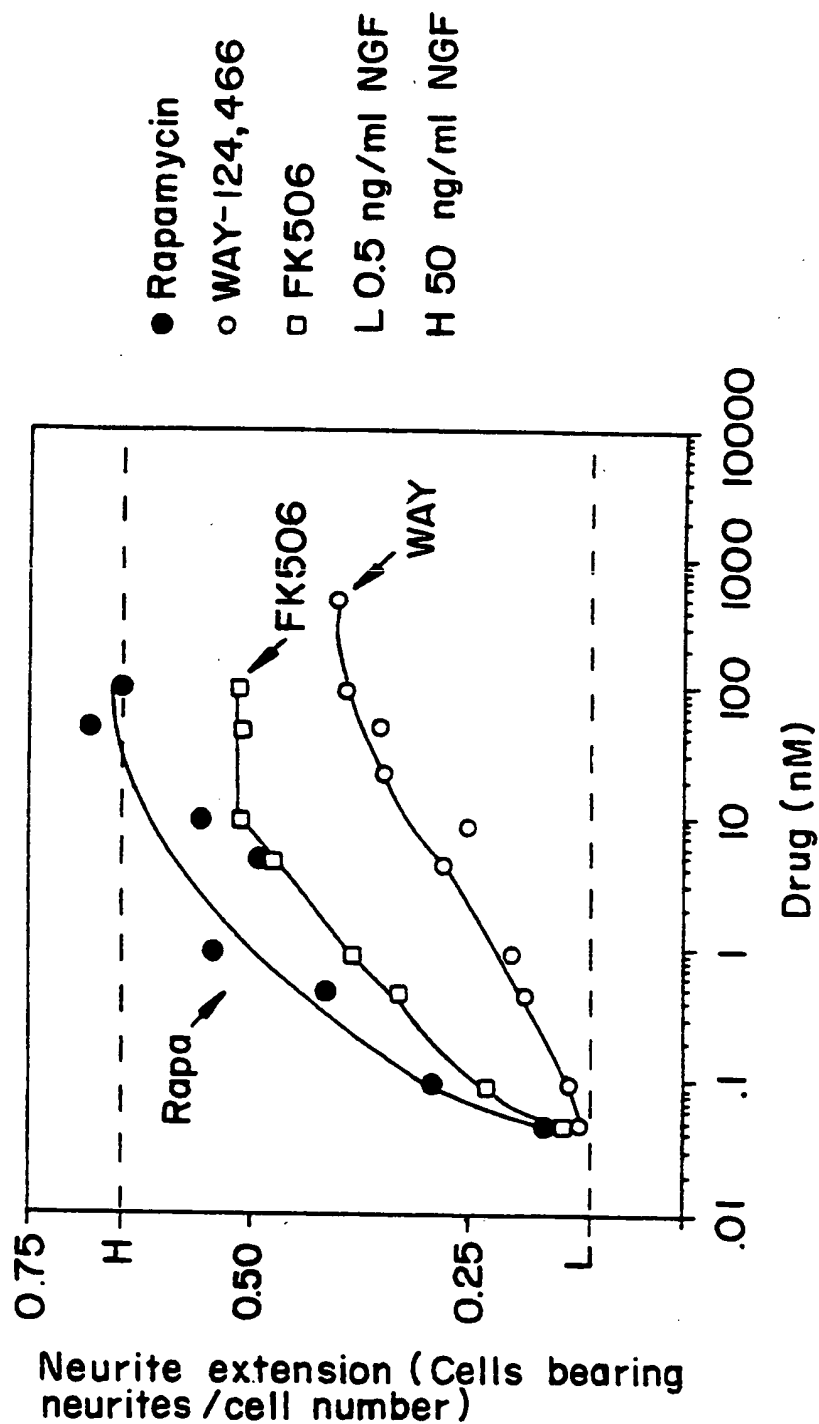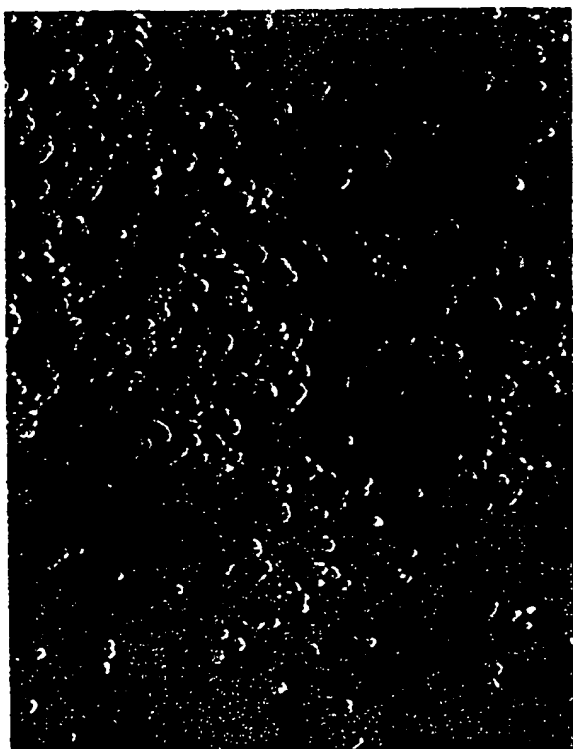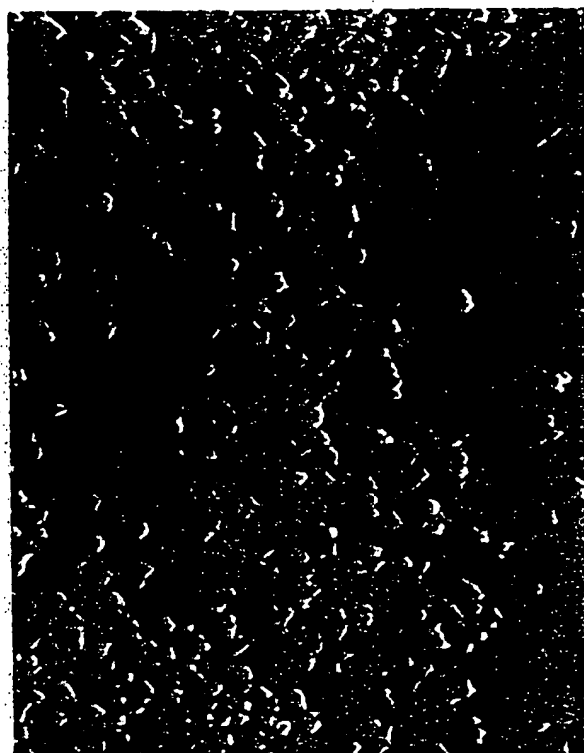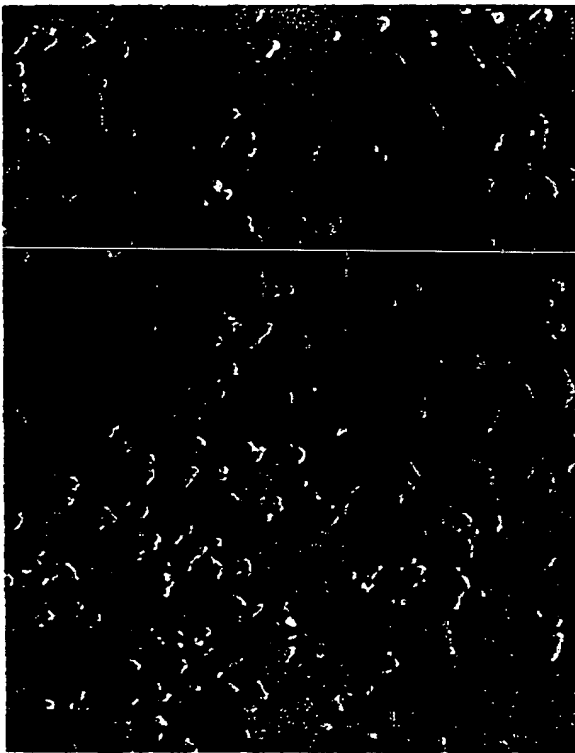
FIG. 16A
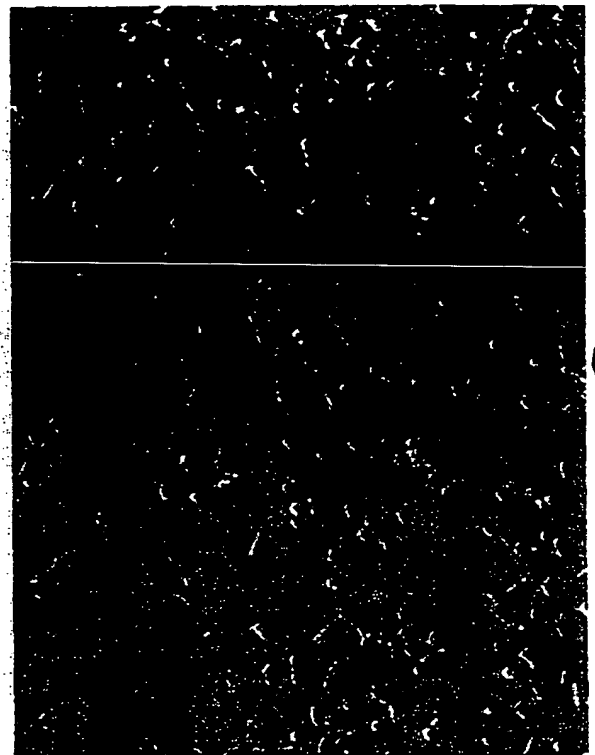
FIG. 16B

FIG. 16C
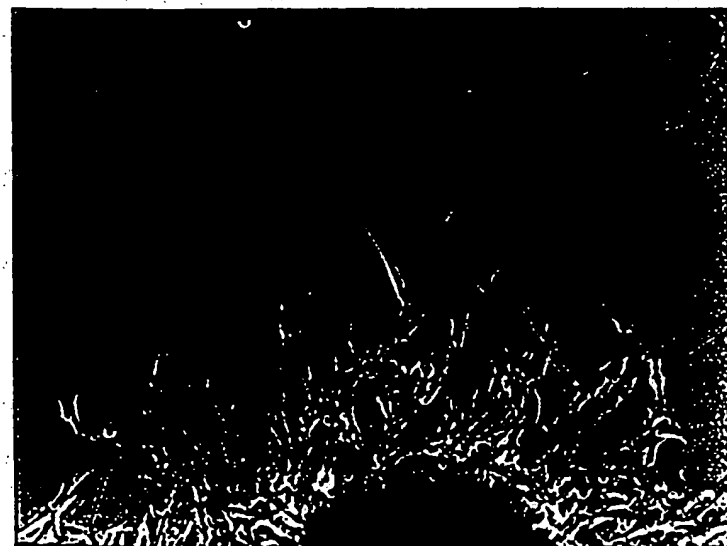
FIG. 16D
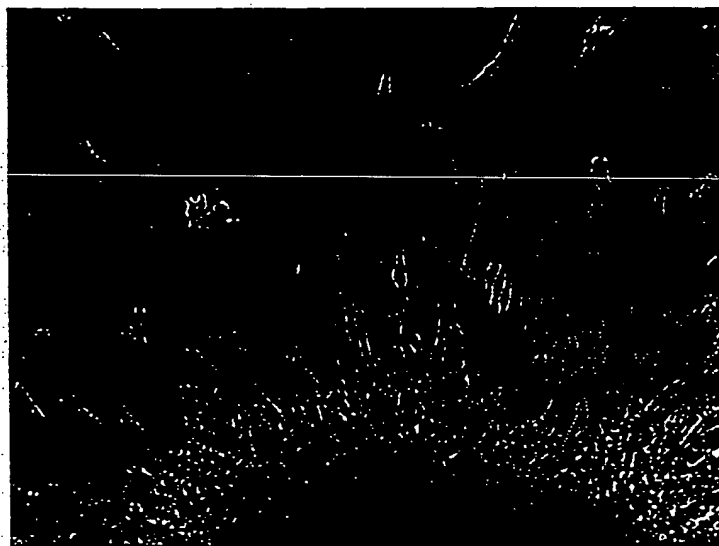
FIG. 16E
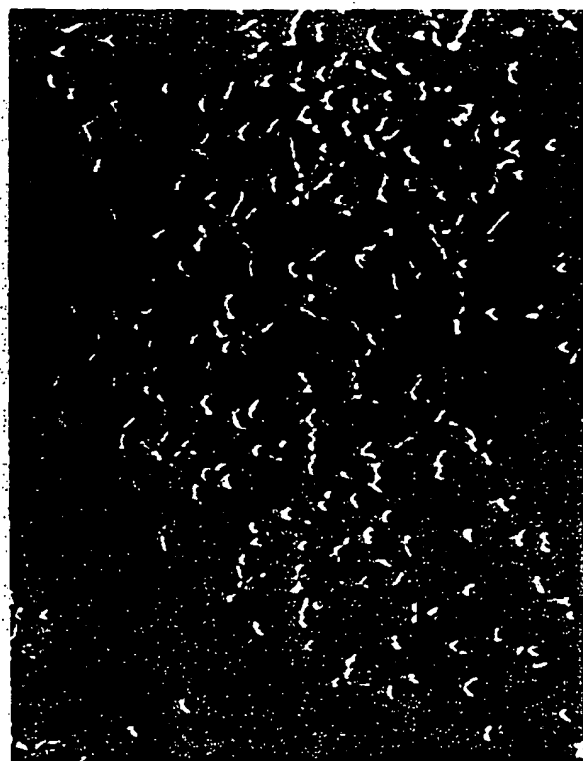
FIG. 16F

FIG. 17A

FIG. 17B

FIG. 17C

FIG. 18
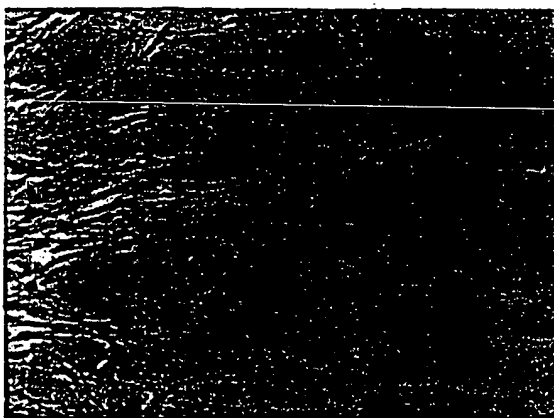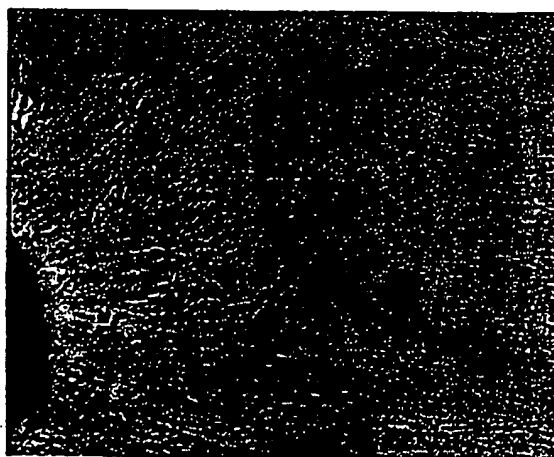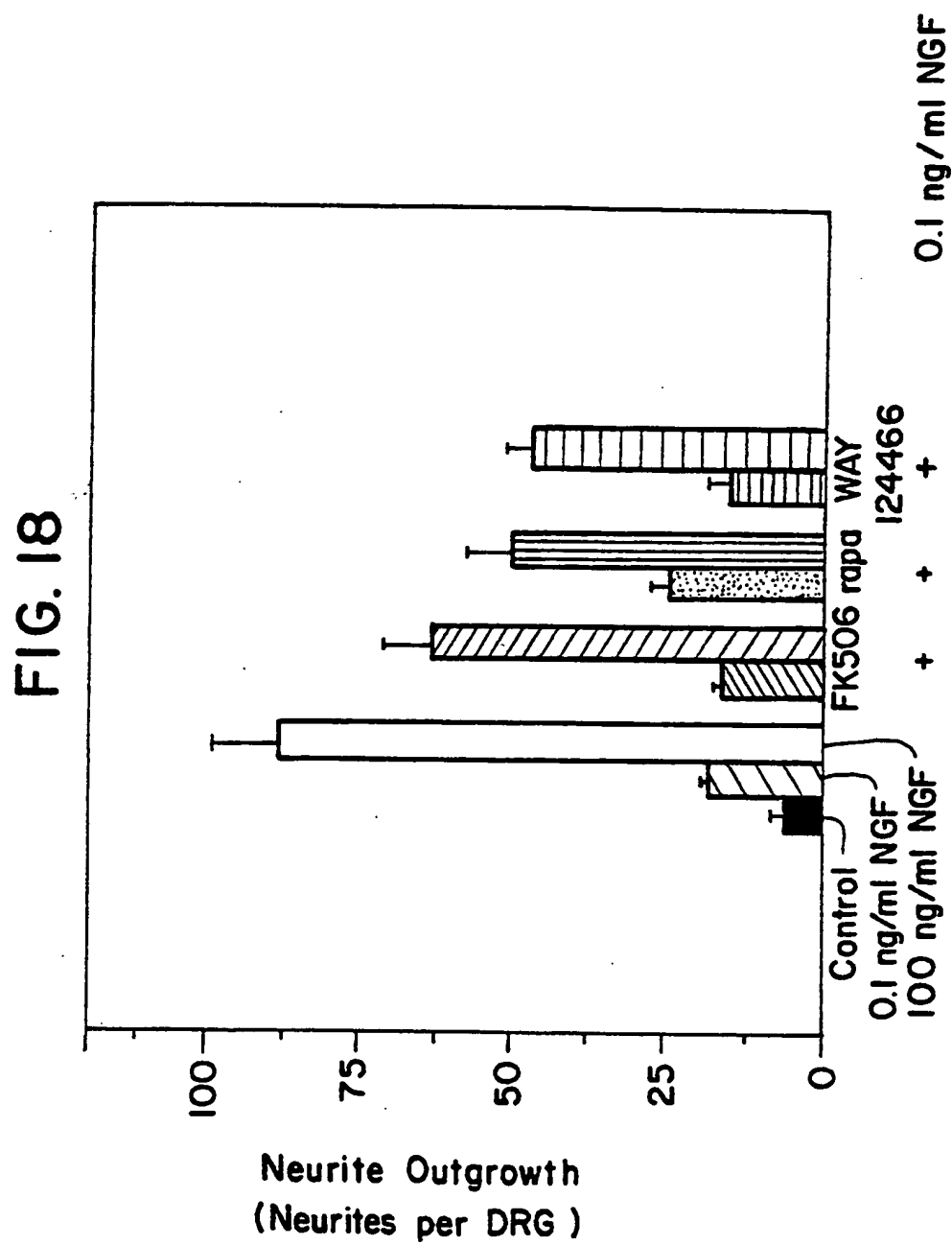
Example 111 Promotes Neurite Outgrowth in Chick Sensory Neurons

100 nM Example 11
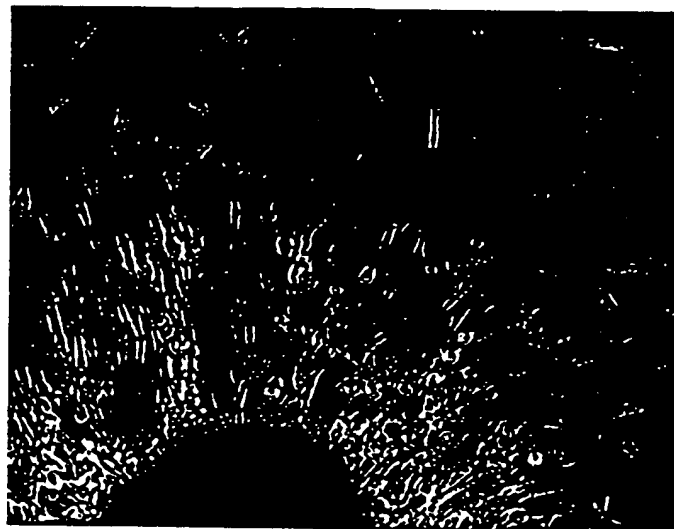
100 pM Example 111

1 pM Example 111

FIG. 19

Example 17 Promotes Neurite Outgrowth in Chick Sensory Neuronal Cultures
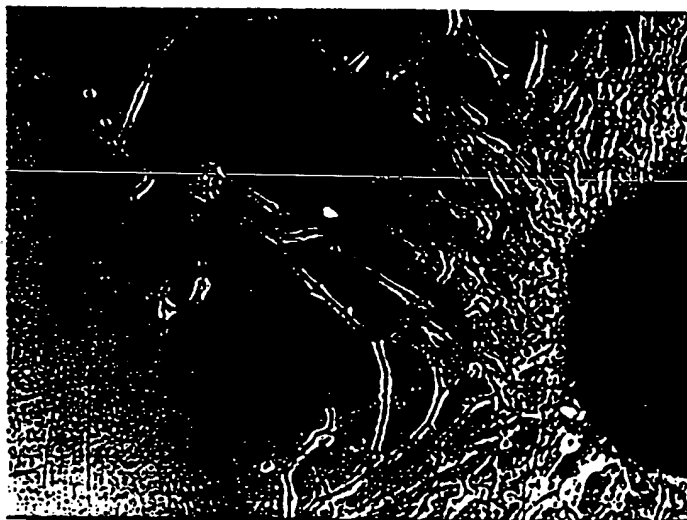

100 nM Exampl 17
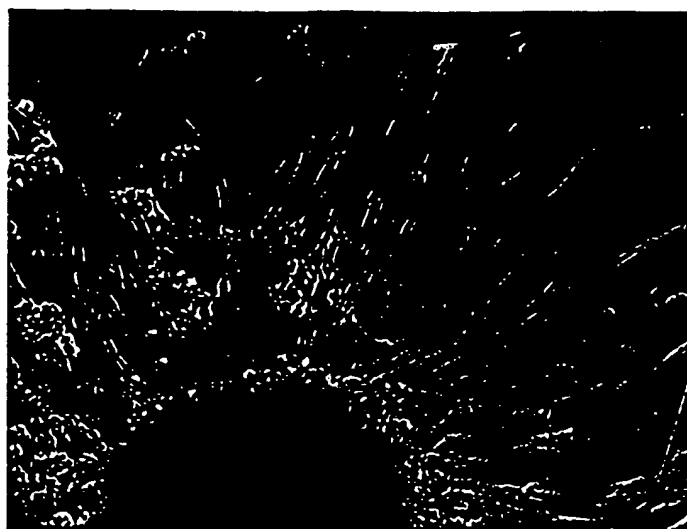
100 pM Example 17

1 pM Example 17

FIG. 20

Example 102 Elicits Neuronal Outgrowth from Chick Sensory Neurons

100 nM Example 102

100 pM Example 102

1 pM Example 102

FIG. 21

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/495, 31/50, 31/44, 31/445
US CL :514/248, 293, 300, 318, 326, 330, 302
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/248, 293, 300, 318, 326, 330, 302

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN(REGISTRY(STRUCTURE), CA, BIOSIS,MEDLINE,USPATFULL, DRUGU)
search terms: Alzheimer's Disease, Parkinson's Disease, amyotrophic lateral sclerosis, als, neuropathies, brain damage, neurodegeneration, stroke, neural regeneration, nerve growth

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 120, No. 25 issued 20 June 1994, Lyons et al, "Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia" see page 48, column 1, abstract no. 315423r, Proc. Natl. Acad. Sci. U.S.A., 91(8), 3191-3195, see entire document.	1-56
X	Chemical Abstracts, Volume 121, No. 19 issued 07 November 1994, Sharkey et al, "Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischemia" see page 95, column 1, abstract no. 221398e, Nature, 371(6495), 336-339, see entire document.	1-56

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 AUGUST 1996

Date of mailing of the international search report

30 SEP 1996

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Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.

PCT/US96/09561

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 121, No. 19 issued 07 November 1994, Gold et al, "The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury", see page 105, column 2, abstract no. 221678w, Restor. Neurol. Neurosci., 6(4), 287-296, see entire document.	1-56
X	Chemical Abstracts, Volume 122, No. 10 issued 06 March 1995, Kelly et al, "Macrolide compositions for treatment of amyotrophic lateral sclerosis", see page 659, column 1, abstract no. 114965m, Brit. UK Pat. Appl. GB 2,279,006, pages 1-14, see entire document.	1-56